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CROSSED IMMUNOELECTROPHORETIC ANALYSIS OF *NEISSERIA MENINGITIDIS* ANTIGENS AND OF CORRESPONDING ANTIBODIES IN PATIENTS WITH MENINGOCOCCAL DISEASE

GERD EVA HOFF and NIELS HØIBY

Department of Infectious Diseases, Rigshospitalet, and Departments of Clinical Microbiology at Rigshospitalet and Hvidovre Hospital, Statens Seruminstitut, Copenhagen, Denmark

Hoff, G E & Høiby, N Crossed immunoelectrophoretic analysis of *Neisseria meningitidis* antigens and of corresponding antibodies in patients with meningococcal disease. Acta path. microbiol scand Sect. C, 86 1-9, 1978

Sixty-three different antigens of *Neisseria meningitidis* groups A, B and C were demonstrated by means of crossed immunoelectrophoresis against rabbit antiserum The group-specific polysaccharides A and C were identified in the immunoprecipitate pattern The majority of the remaining antigens were common to all three groups of meningococci A clearcut rise in antibodies was found in all of 27 patients with meningococcal meningitis or septicaemia These antibodies were directed against both group-specific antigens and common antigens No such rise was seen among the 22 patients with purulent meningitis of non-meningococcal etiology Altogether 15 precipitins were detected in the patients with meningococcal disease Nine of these antibodies could be identified by comparison with the rabbit antiserum The antibody rise was semiquantified by means of a scoring system The rise in the precipitin score of antibodies against common antigens in patients infected with group B meningococci was significantly higher than in patients infected with group A meningococci

Key words *Neisseria meningitidis*, immunoelectrophoresis, serum antibodies

Gerd Eva Hoff Department of Clinical Microbiology at Rigshospitalet, Afsnit 7806, Tagensvej 18, DK-2200 Copenhagen N Denmark

Received 10 vi 77 Accepted 17 viii 77

Immunity to meningococcal infection is due to circulating bactericidal antibodies against meningococci (11, 12, 32) These protective antibodies may be directed against a) the group-specific polysaccharide antigens, b) the protein serotype antigens which are shared among many strains of pathogenic meningococci (28), and c) other common antigens

Infection with *N meningitidis* group A or C or vaccination with the corresponding group-specific capsular polysaccharide gives rise to protective antibodies (9, 11, 12, 13, 14, 15, 16, 32, 36). Vaccination with group B polysaccharide is less protective, since the group B polysaccharide is a poor immunogen (17, 40), and since the antibodies that do develop are not bactericidal (38) Therefore, within group B it appears to be primarily the serotype antigens that elicit bactericidal antibodies (8, 28, 31), in particular the serotype 2 antigen which is the most prevalent in clinical isolates (7) Carrier state with meningococci, groupable or non-groupable, also gives rise to heterologous bactericidal antibodies (12, 33), although serotype 2 is rarely found in strains isolated from carriers (7). Accordingly, common meningococcal antigens shared by both groupable and non-groupable meningococci may be able to induce protective antibodies, and such common antibodies possibly arise in all patients suffering from meningococcal disease.

By means of crossed immunoelectrophoresis, which is a method with a high resolution power, it is possible to differentiate the broad antibody

response arising during infection. We have employed crossed immunoelectrophoresis with intermediate gel (CIWIG) in an analysis of meningococcal antigens precipitated by antibodies raised in rabbits. The antibody response in patients with meningococcal disease was compared with that of the rabbit serum. The purposes were: 1) to identify group-specific meningococcal antigens, serotype antigens and common meningococcal antigens, and 2) to quantify and possibly identify the corresponding human antibodies in patients suffering from meningococcal disease or other infections presenting similar clinical symptoms.

MATERIALS AND METHODS

Preparation of a Polyvalent Reference Meningococcal Antigen (Ref-ag)

Three strains of meningococci representing groups A, B and C were used for production of a reference antigen preparation (group A: ATCC 13077, group B ATCC 13090 and group C: ATCC 13102, kindly supplied by Dr. *Inga Lind*, Statens Seruminstitut, Copenhagen) The serotypes of these strains are not known

The three strains were cultivated separately on solid Bacto ®–Mueller-Hinton medium at 35° C in approximately 5 vol per cent CO_2 for 48 hours, the culture was scraped off the plates, and each strain was suspended in sterile distilled water The proportion between bacteria (wet weight) and water was adjusted to 1 g.1 ml

The bacteria were exposed to ultrasonic disintegration for 3 × 45 seconds at 20,000 cps using a Rapidis 300 W, 19 mm sonde with 9 5 mm tip cooled in ice water The disintegrated products from each strain was centrifuged for 1 hour at 48,200 *g* (max value) at 4° C. This procedure was repeated with the resulting supernatants. The final supernatants were passed through 0 22 μm Millipore® filters

The colloid concentration of the supernatants was estimated by refractometry (AO Instrument Company, Scientic Instrument Division, Buffalo, New York) with human immunoglobulin in distilled water as standard

Ref-ag batch 1 The supernatants from groups A, B and C were mixed The colloid concentrations per litre were 49 6, 41 6 and 18 7 g, respectively This mixture was enriched with polysaccharide A (Merieux A Vaccine Lot 557) to a final vaccine concentration of 10 mg/l before use in immunization Polysaccharides B and C were not available when this antigen was produced

Ref-ag batch 2 Since this batch was used also in electrophoresis, all the supernatants were diluted to a colloid concentration of 10 g/l, in order to avoid favouring any of the antigens in the groups Equal volumes were mixed and Merieux A Vaccine Lot 557 was added as described above The colloid concentration of batch 2 was 10 g/l and the protein concentration 6 7 g/l (Kjeldahl analysis kindly performed by *Wiebke Ramhøj*, Statens Seruminstitut, Copenhagen)

Preparation of a Purified, Concentrated, Polyvalent Rabbit Reference Antibody (Ref-ab) Preparation

Ten rabbits were injected intradermally with Ref-ag in Freund's incomplete adjuvant and bled according to the schedule of *Harboe & Ingild* (19). Each animal received 100 μl Ref-ag per injection. Ref-ag batch 1 was used for the first four injections and Ref-ag batch 2 for the following monthly booster vaccinations (19) Sonicated culture medium was used as immunogen in three rabbits, which were immunized and bled according to the schedule described above

Serum samples of bleedings 2–7 and 8–13 were pooled separately. IgG and IgA were purified and concentrated *a.m Harboe & Ingild* (19). The immunoglobulins made up about 95 per cent of the final protein preparations The protein concentration of Ref-ab pools 2–7 and 8–13 determined by refractometry was 33 6 and 24.0 g/l, respectively.

Anti-group B polysaccharide antiserum (Horse Lot 46) was obtained by the courtesy of Dr. *J B Robbins*, Bureau of Biologics, Food and Drug Administration, Bethesda, Md, U S A

Immunoelectrophoretic Methods

Crossed immunoelectrophoresis with intermediate gel (34) was performed on 5 × 5 cm glass plates using agarose gel (Indubiose A 37, Batch 5388, l'Industrie Biologique Française S A , Gennevilliers, France) and barbital buffer, pH 8 6, r/2 = 0.02 (37) In some experiments, 3 per cent polyethyleneglycol (PEG) 6000 Merck was added to the barbital buffer to increase the sensitivity of the method.

Antigen. 2 μl Ref-ag batch 2 was run in first dimension at 10 V/cm for approximately 30 minutes standardized by a bromphenol blue albumin marker Second dimension electrophoresis was run at 2 V/cm overnight Antibodies in second dimension reference gel 1) 200 μl Ref-ab pool 2–7 in 1 9 ml (13 3 μl/cm²) or 2) 75 μl Ref-ab pool 8–13 in 1 87 ml (5 μl/cm²) Intermediate gel 200 μl saline or serum in 0 8 ml (33 3 μl/cm²) When anti-group B polysaccharide was used, 300 μl of this serum was incorporated in the reference gel

The human precipitins were identified in relation to the Ref-ag/Ref-ab system (3) The quantification of the precipitins in human serum (2) was carried out by comparing the area included by each precipitate in the intermediate gel containing human serum with the corresponding areas in 11 standard plated with Ref-ab pool 2–7 in the intermediate gel (64 μl/cm² and 2-fold dilutions thereof) In this way the titres of the human antibodies were expressed in realtion to the concentration of Ref-ab in the standard plates (34) from titre 1 ≦ 1/16 μl/cm² to titre 12 > 64 μl/cm²

The precipitin score for each patient serum was calculated according to *Axelsen* et al (4) with slight modifications concerning unidentified precipitins. Briefly, each precipitate scored 1 point for being present and an additional ½ point for each step increase in titre

(*e g* a precipitate with titre 1 scored 1 point, a precipitate with titre 2 scored 1½ points, etc) Each unidentified precipitate was given the mean score of the identified precipitins obtained with the same serum

Crossed-line immunoelectrophoresis according to *Krøll* (29) was applied in order to analyse and compare the two Ref-ag batches One µl Ref-ag batch 1 was run against 200 µl Ref-ab pools 2–7 and 8–13, respectively, in 1.9 ml (13 3 µl/cm²) and 200 µl saline or Ref-ag batch 2 in 0 8 ml intermediate gel (33 3 µl/cm²)

Crossed-line immunoelectrophoresis was also used to analyse and compare the antigens from the individual strains of Ref-ag batch 2 The Ref-ag was run against Ref-ab in several different concentrations and with saline or the antigen in question in the intermediate gel in concentrations of 600 µl in 0 8 ml (100 µl/cm²) and in 2-fold dilutions thereof Purified polysaccharide A (vaccine Merieux) and polysaccharide C (vaccine MSD) were also incorporated in the intermediate gel in a concentration of 200 µl in 0 8 ml intermediate gel (33 3 µl/cm²)

CIWIG was used to analyse and compare the two Ref-ab pools 2 µl of Ref-ag batch 2 was run against 200 µl Ref ab pools 2–7 and 8–13, respectively, in 1 9 ml (13 3 µl/cm²) and with 200 µl of the other Ref-ab pool in 0 8 ml intermediate gel (33 3 µl/cm²)

For comparison, the two Ref-ab pools were mixed in different quantities, and 2 µl Ref-ag batch 2 was then run against 200 µl mixed Ref-ab in 2 8 ml (10 µl/cm²)

Immunoelectrophoresis according to *Grabar & Williams* (18) of 10 µl Ref-ag batch 2 against 100 µl Ref-ab pool 8–13 at pH 8 6 in agarose gel showed at least three antigens migrating towards the cathode and therefore escaping detection in the present study, where conventional crossed immunoelectrophoresis detecting only the anodically migrating antigens was used

Human Sera

Sera from the following patients were examined for precipitating meningococcal antibodies

a) 27 patients with meningococcal disease (26 with meningitis and one with septicaemia) verified by culture, antigen detection (20) or microscopy 20 patients were infected with group A and 7 patients with group B meningococci

b) 9 patients with purulent meningitis or septicaemia with known bacterial etiology *S pneumoniae* (5 patients), *S aureus* (2 patients), *E coli* (1 patient) and *K pneumoniae* (1 patient) *S pneumoniae* included types 6A, 8 11 and 23F, while the other bacteria were not typed

c) 13 patients with purulent meningitis where no bacterial diagnosis was established

At least two serum samples were obtained from each patient, one on admission and one after 2 weeks The sera were stored at −20° C with 15 mM/l NaN_3 added

Statistics

Regression correlation (»least square method«) and Mann-Whitney's rang sum test were used (6)

RESULTS

Analysis of Meningococcal Antigens

A reference plate with enumerated precipitates and a corresponding drawing are shown in Fig. 1 (A + B). 63 precipitates were detactable in the Ref-ag preparation, 48 of which were regularly visible. The remaining precipitates could only be detected when the Ref-ag/Ref-ab ratio was changed or 3 per cent polyethyleneglycol was added to the gel.

Control experiments with Mueller-Hinton broth (Bacto) showed that none of the antibodies reacted with culture medium components Rabbits immunized with culture medium did not produce any detectable antibodies

Sera obtained from the rabbits before immunization contained antibodies against antigen no. 19 (titre 1–5) and no. 22 (titre 1) Using different Ref-ag/Ref-ab ratios, it was also possible to demonstrate low-titre antibodies against antigens nos. 4, 5, 46 and 55 in pre-immune sera from some of the rabbits.

No differences in antigen composition could be revealed when the two Ref-ag batches were compared. The three group-specific polysaccharides, A, B and C, were present in the Ref-ag, but in order to demonstrate polysaccharide B, the anti-group B antiserum had to be used in the reference gel. When the three meningococcal strains of Ref-ag batch 2 were compared, only the group-specific polysaccharide antigens (precipitates nos 2 and 7 representing the group A and group C polysaccharide antigens and the group B polysaccharide which avoids detection in the ordinary Ref-ag/Ref-ab pattern) were specific for the individual strain, whereas the remaining antigens were common. However, a few faint precipitates may be strain-specific and experiments are in progress to investigate the participating antigens.

No qualitative differences were revealed between the two Ref-ab pools

Analysis of Meningococcal Antibodies

Antibodies against meningococci were present in sera from all three groups of patients. The mean level of the precipitin score of first sample sera of the three groups of patients was similar (6.3–8 0) and close to that of normal persons, though the range was somewhat greater in meningococcal infections (1 0–14.7) than in the two other groups of patients (3 5–10 6) (21) During the infection, only the patients with meningococcal disease produced a signigicant antibody response against meningococcal antigens, this being demonstrated as an increase in the number of precipitates and in antibody titres against Ref-ag (Fig 2).

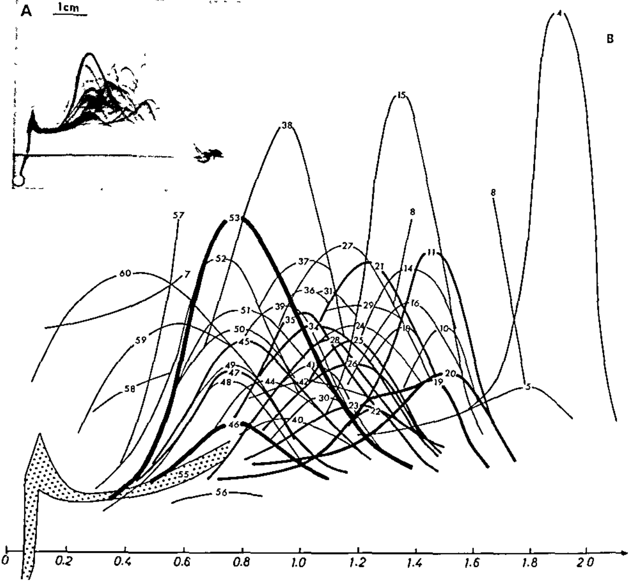


Fig. 1 The meningococcal reference pattern. A Crossed immunoelectrophoresis of Ref-ag against Ref-ab. Saline in the intermediate gel Anode to the right in the first dimension and at the top in the second dimension electrophoresis Staining Coomassie Brilliant Blue B Drawing of A and enumeration of the precipitates An arbitrary scale of migration velocity relative to human serum albumin (1 0) is indicated below

The increase of precipitin score in the three groups of patients is shown in Fig. 3. A rise in precipitin score of 3 or more was seen in patients with meningococcal infection, while there was a lower or no rise in the two other groups If a rise in precipitin score of 3 or more denotes a positive test, then the predictive value of a positive test in identifying patients with meningococcal infection is 27/27 = 100 per cent (95 per cent confidence limits 87–100 per cent), and the predictive value of a negative rest as regards identification of patients without meningococcal infection is 22/22 = 100 per cent (95 per cent confidence limits 85–100 per cent) (6, 39). Altogether, precipitins against 15 different meningococcal antigens were found in the patients' sera. Table 1 shows the prevalence of the identified precipitins. Precipitins against three antigens (nos. 19, 22, 55) were found in nearly all sera. At least six different, unidentified precipitins were found in 15 out of 27 sera from meningococcal patients and one of these also in four sera from the two other groups of patients

In the group of patients with verified group A meningococcal infection, 17 of the 20 patients developed antibodies against group A polysaccharide, whereas none of the seven patients infected with group B meningococci developed group-specific antibodies which could be detected with the reference system. However, low-titred antibodies against the group-specific antigen could be revealed in five of the seven patients infected with group B meningococci, using an anti-group B antiserum in

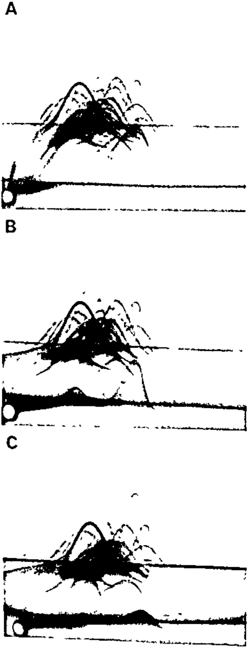


Fig 2 Crossed immunoelectrophoresis of meningococcal Ref ag against meningococcal Ref-ab with intermediate gel containing serum from a patient with meningococcal disease A Serum taken on admission to hospital B Serum taken after one week. C. Serum taken after two weeks Seven precipitates are seen to develop, five of which were identified

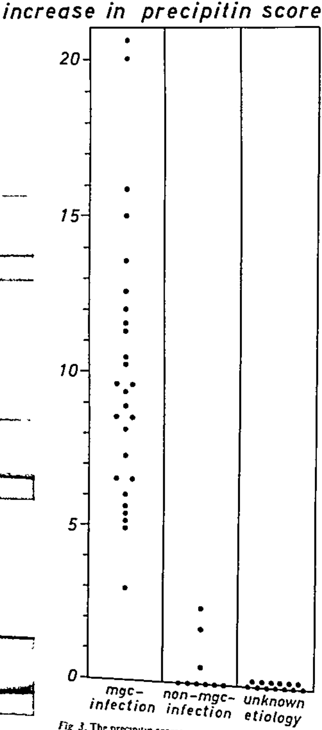


Fig 3. The precipitin scores in paired sera from the three groups of patients A rise in precipitin score of three or more was seen in all patients with meningococcal infection in contrast to patients without meningococcal infection Mgc -infection = meningococcal meningitis or septicaemia Non-mgc -infection = meningitis or septicaemia of different bacterial etiology Unknown etiology = purulent meningitis of unknown etiology.

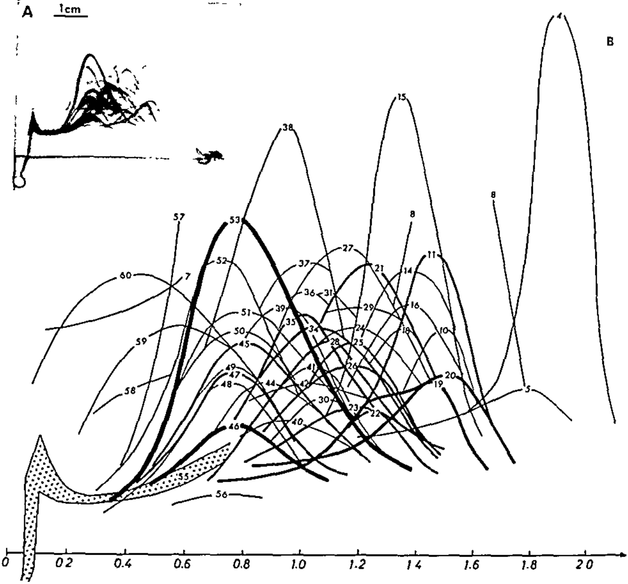


Fig. 1 The meningococcal reference pattern A. Crossed immunoelectrophoresis of Ref-ag against Ref-ab Saline in the intermediate gel Anode to the right in the first dimension and at the top in the second dimension electrophoresis Staining Coomassie Brilliant Blue B Drawing of A and enumeration of the precipitates An arbitrary scale of migration velocity relative to human serum albumin (1 0) is indicated below

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TABLE 1. *Prevalence (per cent) of Precipitins Identified in Sera from Patients*

Precipitin no	2[a]	4	19	20	22	23	45	48	55
Meningococcal meningitis or septicaemia (27 patients)	63	26	100	4	100	7	15	70	96
Meningitis or septicaemia of different bacterial etiology (9 patients)	0	0	100	11	100	0	0	33	100
Purulent meningitis of unknown etiology (13 patients)	0	0	100	0	100	0	0	8	92

[a] Antibody against group A polysaccharide

antibody response included both group-specific antibodies (A and B) and antibodies against common antigens The prevalence of group-specific antibodies in these patients was similar to results obtained with other methods (1) However, some patients failed to develop detectable group-specific antibodies during infection. This could be due to lack of sensitivity of the CIWIG method or lack of development of group-specific antibodies, since immunological tolerance to other polysaccharides has been described (24, 30)

It is interesting that, in spite of a weaker response to the group-specific antigen, patients infected with group B meningococci had a stronger response to common antigens than the group A infected patients The reason for this is not known.

In contrast to *Holten et al* (23), *Reller et al* (33) found that colonization with non-groupable meningococci enhanced bactericidal activity in serum against strains isolated from cases of meningitis of groups A, B, C and Y, due to the appearance of non-group-specific antibodies These antibodies may be directed against serotype antigens or common antigens The prevalence of serotype 2 antigen is low in strains from carriers but high in strains from patients It is not likely therefore, that the enhanced bactericidal activity against pathogenic strains is due solely to antibodies against serotype 2 antigen, but rather to antibodies against common antigens (7, 10)

Antibodies against the common antigens 19, 22 and 55 appeared to develop in all the meningococcal patients However, these antibodies are found also in normal persons (21) It is not known wether the initial titres of these antibodies were too low to be protective, or whether the antibodies are not protective Furthermore, mucosal antibodies in the respiratory tract, which presumably play a protective role, were not investigated in the present study.

The common antigen no 19 is present in many gram-negative bacteria (22, 25, 27). Antibodies against this antigen may thus be induced by a number of bacteria other than meningococci (26), and therefore the presence of these antibodies does not necessarily indicate previous immunization with meningococci.

Antigenaemia was suggested in one patient who subsequently produced antibodies against these antigens The »free« antigens in serum increased the area of two of the reference precipitates (Fig. 4) These changes are interpreted as being due to the presence of partial cross-reactive antigens (25), *i.e* antigens possessing only some of the antigenic sites of the meningococcal antigen in question. The remaining antigenic sites of these »free« antigens could be blocked by the patient's antibodies, since antibodies against these antigens can be found in normal human serum (21) These »free« antigens may therefore, in fact, be immunocomplexes

The degree of cross-reactions between meningococci and the other pathogenic bacteria studied was not very extensive. Two of the three patients showing a minute rise in precipitin score were infected with *E. coli* and *K pneumoniae*. This rise was caused by an increase in antibody titre against antigen no. 19, which cross-reacts with antigens from many gram-negative bacteria (22, 25, 27) Meningococcal disease can be distinguished reliably from other similar infecuous diseases by means of the antibody response in paired sera analysed by the CIWIG method. Furthermore, since this method permits identification of antibody specificities and detection of free antigens, it is a valuable tool in clinical situations.

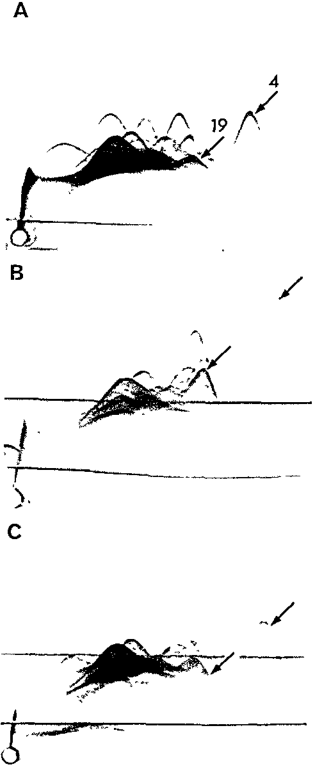


Fig 4 Crossed immunoelectrophoresis of meningococcal Ref-ag against meningococcal Ref-ab with an intermediate gel containing saline or serum from a patient with meningococcal septicaemia. Arrows at precipitates no 4 and no. 19 A Control plate with saline B Serum taken on admission to hospital The area of precipitate no 4 and no 19 is increased C Serum taken after two weeks Precipitate no 19 shows a slight degree of inward feet indicating antibodies in the intermediate gel-serum against antigen no 19.

the reference gel. When only the rise in precipitin score of antibodies against common antigens was considered, this was signigicantly higher in the seven group B patients (mean 9.0, range 5.0–20 0) than in the 20 group A patients (mean 5.2, range 0.9–13.5) ($p<0.05$). The development of unidentified common precipitins was mostly responsible for this difference.

There was a positive correlation between the numbers and titres of identified precipitins both in first sample sera (r: 0.83, $p<0.01$) and in second sample sera (r: 0.74, $p<0.01$). It was decided therefore, to use the mean score of the identified precipitins of a given serum when evaluating the unidentified precipitins of that serum.

In one patient with meningococcal septicaemia, the serum appeared to contain free meningococcal antigens. The first sample serum contained presumably two antigens (nos. 4 and 19), since the area of the two precipitates had increased (Fig. 4) The second sample serum showed antibodies to four antigens, and no free antigens could be detected (Fig. 4)

DISCUSSION

N. meningitidis is a serologically heterogeneous species. At least nine group-specific antigens and 11 type-specific antigens have been demonstrated (7), whereas common antigens have not been studied thoroughly. However, the CIWIG method employed in the present study increased the number of detectable meningococcal antigens to 63. Similar experiences were obtained when CIWIG was applied to studies on the antigen structure of other microorganisms (5)

In order not to lose information, this first study of meningococci by means of CIWIG made use of a sonicated antigen sample from three serogroups representing 90–95 per cent of pathogenic meningococci (16). Inclusion of other groupable meningococci (groups X, Y, Z, D, 29-E, 135) in the antigen preparation and use of detergents during antigen preparation might increase further the number of detectable meningococcal antigens (35).

The majority of the antigens of the Ref-ag preparation were common antigens, but two group-specific and possibly also a number of type-specific antigens were also present. However, the latter antigens require further investigation, because the corresponding precipitates were very faint. No precipitins to group B polysaccharide were observed, in accordance with the reported low immunogenicity of this antigen (17, 40).

During meningococcal infection, an antibody response was seen in all patients examined. The

TABLE 1. *Prevalence (per cent) of Precipitins Identified in Sera from Patients*

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Purulent meningitis of unknown etiology (13 patients)	0	0	100	0	100	0	0	8	92

[a] Antibody against group A polysaccharide

antibody response included both group-specific antibodies (A and B) and antibodies against common antigens. The prevalence of group-specific antibodies in these patients was similar to results obtained with other methods (1). However, some patients failed to develop detectable group-specific antibodies during infection. This could be due to lack of sensitivity of the CIWIG method or lack of development of group-specific antibodies, since immunological tolerance to other polysaccharides has been described (24, 30).

It is interesting that, in spite of a weaker response to the group-specific antigen, patients infected with group B meningococci had a stronger response to common antigens than the group A infected patients. The reason for this is not known.

In contrast to *Holten et al.* (23), *Reller et al.* (33) found that colonization with non-groupable meningococci enhanced bactericidal activity in serum against strains isolated from cases of meningitis of groups A, B, C and Y, due to the appearance of non-group-specific antibodies. These antibodies may be directed against serotype antigens or common antigens. The prevalence of serotype 2 antigen is low in strains from carriers but high in strains from patients. It is not likely therefore, that the enhanced bactericidal activity against pathogenic strains is due solely to antibodies against serotype 2 antigen, but rather to antibodies against common antigens (7, 10).

Antibodies against the common antigens 19, 22 and 55 appeared to develop in all the meningococcal patients. However, these antibodies are found also in normal persons (21). It is not known wether the initial titres of these antibodies were too low to be protective, or whether the antibodies are not protective. Furthermore, mucosal antibodies in the respiratory tract, which presumably play a protective role, were not investigated in the present study.

The common antigen no. 19 is present in many gram-negative bacteria (22, 25, 27). Antibodies against this antigen may thus be induced by a number of bacteria other than meningococci (26), and therefore the presence of these antibodies does not necessarily indicate previous immunization with meningococci.

Antigenaemia was suggested in one patient who subsequently produced antibodies against these antigens. The »free« antigens in serum increased the area of two of the reference precipitates (Fig. 4). These changes are interpreted as being due to the presence of partial cross-reactive antigens (25), *i.e.* antigens possessing only some of the antigenic sites of the meningococcal antigen in question. The remaining antigenic sites of these »free« antigens could be blocked by the patient's antibodies, since antibodies against these antigens can be found in normal human serum (21). These »free« antigens may therefore, in fact, be immunocomplexes.

The degree of cross-reactions between meningococci and the other pathogenic bacteria studied was not very extensive. Two of the three patients showing a minute rise in precipitin score were infected with *E. coli* and *K. pneumoniae*. This rise was caused by an increase in antibody titre against antigen no. 19, which cross-reacts with antigens from many gram-negative bacteria (22, 25, 27). Meningococcal disease can be distinguished reliably from other similar infectious diseases by means of the antibody response in paired sera analysed by the CIWIG method. Furthermore, since this method permits identification of antibody specificities and detection of free antigens, it is a valuable tool in clinical situations.

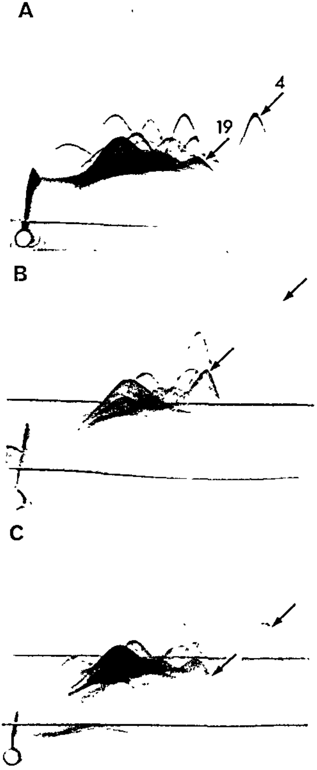


Fig. 4 Crossed immunoelectrophoresis of meningococcal Ref-ag against meningococcal Ref-ab with an intermediate gel containing saline or serum from a patient with meningococcal septicaemia. Arrows at precipitates no. 4 and no. 19. A. Control plate with saline. B. Serum taken on admission to hospital. The area of precipitate no. 4 and no. 19 is increased. C. Serum taken after two weeks. Precipitate no. 19 shows a slight degree of inward feet indicating antibodies in the intermediate gel-serum against antigen no. 19.

the reference gel. When only the rise in precipitin score of antibodies against common antigens was considered, this was signigicantly higher in the seven group B patients (mean 9.0, range 5.0–20.0) than in the 20 group A patients (mean 5.2, range 0.9–13.5) ($p<0.05$). The development of unidentified common precipitins was mostly responsible for this difference.

There was a positive correlation between the numbers and titres of identified precipitins both in first sample sera (r: 0.83, $p<0.01$) and in second sample sera (r: 0.74, $p<0.01$). It was decided therefore, to use the mean score of the identified precipitins of a given serum when evaluating the unidentified precipitins of that serum.

In one patient with meningococcal septicaemia, the serum appeared to contain free meningococcal antigens. The first sample serum contained presumably two antigens (nos. 4 and 19), since the area of the two precipitates had increased (Fig. 4) The second sample serum showed antibodies to four antigens, and no free antigens could be detected (Fig. 4).

DISCUSSION

N. meningitidis is a serologically heterogeneous species. At least nine group-specific antigens and 11 type-specific antigens have been demonstrated (7), whereas common antigens have not been studied thoroughly. However, the CIWIG method employed in the present study increased the number of detectable meningococcal antigens to 63. Similar experiences were obtained when CIWIG was applied to studies on the antigen structure of other microorganisms (5).

In order not to lose information, this first study of meningococci by means of CIWIG made use of a sonicated antigen sample from three serogroups representing 90–95 per cent of pathogenic meningococci (16). Inclusion of other groupable meningococci (groups X, Y, Z, D, 29-E, 135) in the antigen preparation and use of detergents during antigen preparation might increase further the number of detectable meningococcal antigens (35)

The *majority of the antigens of the* Ref-ag preparation were common antigens, but two group-specific and possibly also a number of type-specific antigens were also present. However, the latter antigens require further investigation, because the corresponding precipitates were very faint. No precipitins to group B polysaccharide were observed, in accordance with the reported low immunogenicity of this antigen (17, 40).

During meningococcal infection, an antibody response was seen in all patients examined. The

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FUNCTIONAL ANALYSIS OF LYMPHOID CELLS FROM MOUSE BONE MARROW IN VITRO

BENT RUBIN, M HØIER-MADSEN, and CARSTEN RÖPKE*

*) Anatomy Department A, University of Copenhagen

Immunobiology Laboratory, Statens Seruminstitut, Copenhagen, Danmark

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The present results show that mouse bone marrow (BM) cells responded in vitro against phytohaemagglutinin (PHA), concanavalin A (Con A) and lipopolysaccharide (LPS) In addition, BM cell suspensions contained relatively many cells with ADCC (antibody-dependent cell-mediated cytotoxicity) activity BM cells were fractionated according to various cell surface characteristics The results of these experiments were compared with previously published reports, showing that pre-T and pre-B cells could be separated on Ig anti-Ig columns (Ig = immunoglobulin) The combined results showed that pre-T cells may fractionate together with cells with the following characteristics they displayed ADCC activity and had Fc receptors (FcR) and/or membrane Ig Pre-B cells fractionated with cells with the following characteristics they responded to LPS but had no ADCC activity They were not adherent and/or phagocytic and were Ig⁻, FcR⁻ and C3 receptor (C3R) negative

Key words Mouse bone marrow cells, mitogenic response, ADCC activity.

Bent Rubin, Immunobiology Laboratory, Bloodbank/Blood Grouping Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.

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Recent observations from this laboratory have suggested that, contrary to expectation, BM precursor B cells do not express FcR and/or membrane Ig. BM precursor T cells would seem to express either FcR or membrane Ig or both. Such results would indicate that precursor cells for the B or T lineage are different, and that the surface markers on precursor cells and on mature effector cells within a given cell lineage may differ or change (15, 17)

BM cells respond in vitro against T cell mitogens PHA or Con A (4) and the B cell mitogen LPS (8) Furthermore, lymphoid cells in the BM can kill antibody coated target cells (Ab-CRBC = antibody coated chicken erythrocytes) in vitro (3) Thus, non-fractionated and fractionated (non-specific adherence, phagocytosis, affinity chromatography) BM cells were assayed for their reactivity in vitro against PHA, Con A, LPS and Ab-CRBC. The aim of the study was to clarify whether pre-B or pre-T cells in bone marrow fractionate together with BM lymphoid cells displaying any of the above-mentioned in vitro functions.

The results suggest that a pre-B cell could be classified as an LPS responding cell which does not express FcR, membrane Ig or C3R. Pre-B cells were not endowed with ADCC activity, and were not adherent or phagocytic as defined by the present methods. Pre-T cells were seemingly not able to react with the T cell mitogen PHA. They would seem to fractionate along with the ADCC effector cells among BM cells.

MATERIALS AND METHODS

Animals Balb/cxCBA F1 hybrid mice of both sexes were bred in our colony and were 6–8 weeks old when used

Mitogens Purified PHA was obtained from Wellcome Research Laboratories (HA17, lot no 8805) Con A from Pharmacia (lot no 3059) and *E coli* 055 B5 LPS from Difco Laboratories (lot. no 503309) Stock

solutions of each mitogen in sterile saline contained 2 mg/ml.

Cultures. Eagles minimum essential medium F13 (Gibco), supplemented with 100 I.U. of penicillin/ml. 10 μg of Gentamicin/ml. 2mM glutamine, 10mM Hepes, and non-essential amino acids, was used as tissue culture medium. In addition, the medium contained 10 per cent foetal calf serum (FCS) (Flow Laboratories lots no. 470055 and 415075 and Gibco/Biocult lot no. 553018) This medium will be called FCS/F13.

Cell suspensions were made aseptically from the thymus, spleen or tibias and femurs. The cells were washed twice in FC3/F13 and the number of trypan blue excluding cells was determined. The cell concentration was adjusted to 10 times the desired concentration. 0.1 ml of this stock solution was added to Falcon plastic tubes (no 2001) prefilled with 1.8 ml FCS/F13 and 0.1 ml of mitogen Mitogen responses were determined in triplicate. All cultures were incubated at 37° C in a humified atmosphere containing 5% CO_2. Optimal conditions for spleen and thymus cell cultures were described previously (10)

Different sources of serum were tested for supporting the growth of BM cells: rat serum(6), guinea pig serum, human serum or FCS. 10% FCS was found to be the best supplementing serum for BM cells in our system. The optimal cell density, mitogen concentration and culture time were also investigated Optimal stimulation indices were obtained by culturing $2 \times 10^5 - 10 \times 10^5$ BM cells/ml for 5 days in the presence of 10 μg/ml of PHA, Con A or LPS. ^{3}H-thymidine was added to the cultures 18 hours before termination Neither the optimal concentrations of mitogens or cells nor the culture time were changed as a consequence of the fractionation procedures employed (see e.g Table 2)

Morphological characterization of cells and their fractionation on columns. The following assays have been described in detail previously: T cells were identified by means of a cytotoxicity assay employing anti-θ antiserum and complement (10), B cells were identified by using EA- or EAC'-RFC complexes (E = sheep erythrocytes (SRBC), A = anti SRBC antibody of 7S or 19S class respectively, C' = C5 deficient mouse serum (10) or by using immunoflurorescense (17), Macrophages were identified by their ability to stain with acridine orange (AO) (5) or to phagocytize Latex particles (9), Affinity columns were made by coating glass beads with either human serum albumin (HSA), mouse immunoglobulin (Ig), HSA anti-HSA or Ig anti-Ig complexes (17, 18)

Adherence to plastic surfaces Lymphoid cells adjusted to 10×10^6 cells/ml were incubated for different times on the surface of Falcon plastic flasks (no 3024) in a volume of 10 ml (Fa) After incubation, non-adherent cells (NA) were shaken off gently, centrifuged and resuspended into the volume reguired (9) Adherent cells were eluted by incubating the flasks with 5 ml of FCS/F13 medium containing 5 mM EDTA for 15 minutes at 37° C The cells obtained by this procedure (not all cells were removed) were washed twice in EDTA-free medium and resuspended into a the volume reguired (AE cells) In some experiments NA cells were further incubated on one or more consecutive plastic flasks; in such cases NA cells were poured directly into the new flasks. Optimal incubation time was 45 minutes If the incubation time was extended for longer times, the number of macrophage-like cells was further reduced, but then also B cells and cytotoxic ADCC effector cells were removed to a significant extent.

Treatment with carbonyl-iron and magnet (Fe) and combination of Fa and Fe techniques. Lymphoid cell suspensions were depleted of phagocytic cells using the Fe technique (5). 20 mg of carbonyl-iron per 10 ml was found to be optimal for specific removal of macrophage-like cells from 20×10^6 cells/ml. More Fe or extended incubation times (more than 30 minutes at 37° C) caused non-specific reduction in the number of T, B or K lymphocytes. However, a more significant depletion of macrophage-like cells without significant depletion of T, B or K lymphocytes was obtained by combining the Fa and Fe techniques. This was done either by first incubating the cells with Fe and then on Fa (Fe + Fa), or by incubating the cells with Fe in a Falcon flask (Fa/Fe) (see Table 1).

Cytotoxicity against antibody coated target cells (ADCC assay). The cytotoxic potential of mouse thymus, spleen or BM cells against antibody coated chicken erythrocytes (Ab-CRBC) was determined as described

TABLE 1. *Content of Macrophages in Spleen Cell Suspensions before and after Fractionation*[a]

Fractionation, technique[b]	Acridine Orange staining cells	Latex particle phagocytizing cells
C	100	100
Fe	9 ± 2 1	9 ± 1.2
Fa	13 ± 5.3	18 ± 6.0
Fe + Fa	3 ± 0 9	5 ± 0.8
HSA-column	21 ± 8 1	29 ± 9 2
Ig-column	19 ± 7.4	23 ± 4.3
HSA anti-HSA column	11 ± 3 0	9 ± 3.6
Ig anti-Ig column	6 ± 0 9	7 ± 0.3

a) Means of 14 experiments. Number of acridine orange staining cells and latex phagocytizing cells in non-fractionated cells *(C) = 7 3% ± 0 8 6 1% ± 0 4 (one* standard deviation), respectively These figures are arbitrarily given the value 100 and the content of macrophages in fractionated cells is calculated in relation thereto

b) Fe = cells treated with carbonyl-iron and magnet. Fa = cells incubated onto Falcon flask for 45 minutes at 37° C, Fe + Fa = cells treated first with Fe and then with Fa. Cells were also passed through columns coated with HSA, -HSA or Ig anti-Ig complexes.

TABLE 2. *Mitogenic Responses of Mouse Spleen Cells Depleted of Adherent/Phabocytic Cells*[a]

Motogen/ml	Spleen cell fractionation[b] C	Fe	Fa	Fe + Fa
10 μg PHA	12,809	11,324	10,296	12,935
1 »	64,946	50,465	42,473	40,879
0 1 »	4,701	8,053	5,895	8,259
10 μg Con A	21,458	14,110	5,113	11,512
1 »	74,579	56,557	45,471	52,426
0 1 »	4,376	19,643	3,082	5,690
100 μg LPS	31,476	27,155	26,505	32,469
10 »	41,476	35,417	34,510	38,272
1 »	24,864	22,780	26,076	26,620
% Recovery	–	79.5	92.7	76 3
% θ pos cells	24 8	47 9	36 2	42.5
% EAC'-RFC	15 9	10 8	13 2	8 5
% AO/Latex pos cells[c]	8 3/6 9	0 9/0 6	1 6/1 5	0 3/0 3

[a]) 10^6 spleen cells/ml culture Incubation time = 3 days Results are expressed as Δ cpm = cpm in mitogen stimulated cultures minus cpm in unstimulated cultures

[b]) See Table 1

[c]) AO = acridine orange staining cells, Latex pos. cells = cells phagocytizing latex particles (See also Table 1)

previously (18) The results are expressed as percentage specific ^{51}Cr release, i e ^{51}Cr released from 7S Ab-CRBC minus ^{51}Cr release from uncoated CRBC by the different kinds of effector cells Culture time was 18 hours and spontaneous release from CRBC was <2 5 per cent Cytotoxicity by thymus, spleen or BM cells in absence of antibody was <5 0 per cent

Statistics The mitogenic cultures were made in triplicate as described Geometric means and SD (one standard deviation) were calculated The results are given as 1) cpm (anti-$\log_{10}$ to the geometric means) 2) Δcpm = cpm in mitogen stimulated cultures minus cpm in unstimulated cultures The SD is not stated in order to make the tables and figures less complex, and because the variation between the three cultures very seldom exceeded 5 per cent 3) SF = stimulation factor = cpm in mitogen stimulated cultures divided by cpm in unstimulated cultures The Student's test was used to determine the degree of significance, if needed

RESULTS

In Vitro Activities of Macrophage Depleted Thymocytes and Splenocytes

Non-fractionated thymus and spleen cells and cells fractionated on a plastic surface (Fa), treated with Fe or with the Fe + Fa combination of the two techniques, were tested for their proliferative responses against PHA, Con A or LPS and for their ADCC activity. In addition, the number of θ positive cells, EA- or EAC-RFC and macrophage-like cells was determined. Table 2 gives the results of one out of five experiments. It may be concluded that depletion of macrophage-like cells by any of the methods used has no dramatic impact on the mitogenic response of spleen cells (13, 14). However, treatment with Fe caused a five-fold reduction in the cytotoxic potential of spleen cells (data not shown) as found by others (6)

The PHA or LPS response of thymocytes was either the same or somewhat enhanced after depletion of macrophage-like cells, whereas the Con A response was significantly reduced. However, the adherent cells (AE) eluted with EDTA were enriched for cells responding to Con A and LPS (Table 3, (2)) Non-fractionated or NA thymocytes displayed no ADCC activity, but AE thymocytes did (Table 4)

Morphological Observations on BM Cells Fractionated by the Different Methods

The mean recovery of live nucleated BM cells after the different fractionation procedures used in the present study can be seen in Table 5. Whereas the adherence/phagocytosis procedures gave recoveries around 80 per cent, the recovery of BM cells

TABLE 3. *Mitogenic Responses of Mouse Thymus Cells Depleted of Adherent/Phagocytic Cells*[a]

Mitogen/ml[b]	Thymus cell fractionation[c]				
	C	Fa-NA	Fa-AE	Fe	Fe + Fa
	3,615	1,288	3,275	1,960	2,044
10 μg PHA	20,994	31,186	22,853	25,497	27,833
1 μg Con A	88,323	32,935	138,587	13,487	10,528
10 μg LPS	2,605	2,822	19,235	3,745	3,187
% Recovery	–	51.3	5 6	90.5	59.2
% EA-RFC	0.86	0.21	3.28	0 47	0.18

a) Mitogenic responses are expressed as cpm per culture This experiment is one of 11 experiments with similar results

b) 5×10^6 cells/ml except Con A cultures ($= 2.5 \times 10^6$/ml)

c) C = non-fractionated thymus cells. Fa = cells incubated on a Falcon flask for 3 hours, (NA) = non-adherent cells which were poured off and (AE) = adherent cells which were eluted with EDTA. Fe = cells treated with carbonyl-iron and magnet, Fe + Fa = cells treated with carbonyl-iron on a plastic flask.

after the column fractionation procedures was about 30 per cent. The number of BM lymphocytes in non-fractionated cell suspensions was about 30 per cent, and this number was somewhat enhanced after Fe treatment and HSA column fractionation (40 per cent). Thus, there was no selective decrease of BM lymphocytes by our fractionation procedures. It would rather seem that non-lymphocytic cells are removed non-specifically, thereby increasing the number of BM lymphocytes. The specific columns would then retain those BM lymphocytes with the equivalent specific surface markers (θ, FcR, C3R or Ig).

TABLE 4 *ADCC Activity of Non-fractionated and Fractionated Thymocytes Compared with that of Spleen Cells*[a]

Effector Cells[b]	% EA-RFC	Cytotoxicity against Ab-CRBC[c]		
		Target effector cell ratio		
		1.4	1 20	1.100
Thymocytes-C	1 2	1 4	2 1	3 9
Thymocytes-NA	0 2	–0 1	–0 4	0 2
Thymocytes-E	6 2	4 4	10 1	16 3
		1 2	1 10	1 50
Spleen cells-C	38 7	12 2	33 4	66 2

a) This experiment is one of 11 experiments with similar results

b) Thymocytes (from C57 × CBA mice) were adsorbed onto Falcon flasks for 3 hours at 37° C/5% CO_2 NA = non-adherent cells (recovery = 61%), AE = adherent cells eluted with 5 mM EDTA (recovery = 3 4%) All cell suspensions were washed three times before ADCC assay

c) Spontaneous release of Ab-CRBC = 3 9%

In Vitro Activities of BM Cells Depleted of Macrophage-like Cells

BM cells were weakly but significantly stimulated by PHA or Con A. They were very significantly stimulated by LPS (Table 6). Removal of Fe

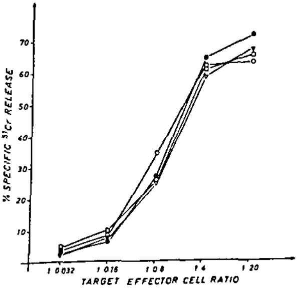


Fig. 1. Cytotoxicity of mouse bone marrow cells against Ab-CRBC before and after removal of adherent and/or phagocytic cells 0 – 0 = non-fractionated. ●–● = Fa, □–□ = Fe, ▽–▽ = Fe + Fa.

TABLE 5. *Morphological Characterization of Bn Cells Subjected to Cell Fractionation*[a]

Fractionation technique[b]	% Recovery	% Lymphocytes	% θ pos cells	% EA-RFC	% EAC-RFC	% Ig pos cells
C	100	30 2	3 8	18 9	22.9	6 1
Fa	82	31.8	4 2	13 7	24 3	–
Fe	90	40 3	3 9	17.6	29.1	–
Fa/Fe	73	38 1	4 5	15 8	30 8	–
Ig	40	37.6	5.1	2.3	5 8	10.2
HSA	37	36 8	3 9	17.5	6 9	8 9
HSA anti-HSA	30	39 2	5 0	0 8	2 8	14 3
Ig anti-Ig	28	42 3	4 1	0 5	0 7	0 2

a) % cells with a given surface marker are calcuted on the basis of the number of viable, nucleated cells in the bone marrow % lymphocytes is determined by differential counts of smears of bone marrow cells stained with May-Grunwald/Gimsa Stain – = not done The results are mean values of 9 experiments

b) Cells were either non-fractionated (C) or fractionated on Falcon flasks (Fa), with carbonyl-iron and magnet (Fe) or with Fe in a Falcon flask (Fa/Fe) or passed through columns coated with HSA, Ig, HSA anti-HSA or Ig anti-Ig complexes

phagocytizing cells had no impact on the responses to PHA or Con A, whereas the LPS response was increased about 2-fold (Table 6). On the other hand, removal of plastic surface adherent cells augmented the responses to PHA or Con A, whereas the LPS response seemed to be reduced slightly (Table 6). Both procedures appear to have little or no influence on the ADCC activity of BM cells (Fig. 1, (3))

In Vitro Activities of BM Cells Subjected to Affinity Column Fractionation

BM cells were fractionated on glass bead columns coated with Ig, HSA, HSA anti-HSA or Ig anti-Ig complexes The HSA or Ig columns should function more or less like the adherence technique described in the previous section, whereas the HSA anti-HSA or Ig anti-Ig columns in addition should remove

TABLE 6. *Effect of Removing Adherent and/or Phagocytic Cells from Bone Marrow Cells on their Subsequent Mitogenic Responses*[a]

Mitogen/ml	Bone marrow cell fractionation[b] C	Fa	Fe	Fe + Fa
–	1,214	1,639	1,317	1,228
10 μg PHA	2,649	4,011	2,724	2,593
» Con A	2,213	4,161	2,160	2,630
» LPS	16,008	13,610	27,442	13,666

a) 5×10^5 bone marrow cells per ml culture Results are expressed as cpm This experiment is one of 9 experiments with similar results

b) See Table 1

TABLE 7 *Summary of the Effect of Affinity Column Fractionation on the Mitogenic Responses of Mouse Bone Marrow Cells*[a]

Mitogen/ml	BM cells fractionated on columns coated with[b] C	HSA	Ig	HSA anti-HSA	Ig anti-Ig
–	(979)	(681)	(560)	(694)	(893)
10 μg PHA	3 288	6,060	4,183	6,119	7,680
» Con A	1,873	1,489	1,571	1,034	696
» LPS	11,808	43,217	44,290	39,989	13,758

a) Mean of five separate experiments 5×10^5 BM cells per ml culture Culture time = 5 days Results are expressed as Δ cpm (See Table 2) cpm in unstimulated cultures is given in brackets

b) C = non-fractionated See Table 1



Fig. 2. Cytotoxicity of mouse bone marrow cells against Ab-CRBC before and after fractionation on affinity columns 0 – 0 = non-fractionated, ●–● = HSA: P; ∇–∇ = Ig: P; □–□ = HSA anti-HSA: P ▲–▲ = Ig anti-Ig:P (see legend to Table 7).

FcR^+/Ig^+ cells, respectively. The column fractionation procedures reduced the Con A responses of BM cells, whereas they augmented the PHA responses non-specifically (Table 7). However, all columns except the Ig anti-Ig column augmented the LPS response about 3-fold Passage through an Ig ant-Ig column resulted in a cell population significantly depleted of FcR^+-, $C3R^+$-, or Ig^+ cells and phagocytic cells (Tables 1 and 5), but nevertheless this cell population does give an LPS response comparable to that of non-fractionated cells (Table 7)

BM cells passed through Ig, HSA anti-HSA or Ig anti-Ig columns showed a very reduced ADCC activity (Fig. 2). BM cells passed through an HSA column showed a cytotoxic potential which was only somewhat reduced (compare results i Fig. 1). The reduction of cytotoxic activity of BM cells passed through Ig columns could mean that the FcR on BM cells have a relatively high avidity for aggregated Ig (some aggregation of Ig during the preparation of the Ig columns cannot be avoided).

DISCUSSION

Two functional activities of BM cells in vitro were investigated before and after fractionation according to adherence/phagocytosis or adsorption onto affinity chromatography columns: 1) Proliferative responses against T cell mitogens PHA or Con A and the B cell mitogen LPS: 2) cytotoxic activity against Ab-CRBC. The results showed that BM cells responded weakly to PHA or Con A and very well to LPS. Removal of adherent cells had no signigicant effect on these responses, whereas removal of phagocytic cells increased the PHA and LPS responses (Table 6). The PHA response was increased after passage through Ig anti-Ig columns (16) but the reduction of Con A response was similar to that seen with spleen or thymus cells (10). The LPS response and the number of Ig^+ lymphoid cells was greatly increased following passage through HSA anti-HSA columns. However, following passage through Ig anti-Ig columns, the LPS response of BM cells was reduced to a level similar to that of non-passed BM cells, whereas the number of Ig^+ lymphoid cells was reduced by more than 95 per cent. The cytotoxic potential of BM cells against Ab-CRBC was not changed after removal of adherent or phagocytic cells, but was much reduced following passage through columns coated with aggregated Ig or antigen-antibody complexes (Fig. 1 and 2).

One purpose of the present study on the in vitro functions of BM cells before and after fractionation was to compare the results of such studies with the results obtained with fractionated BM cells in vivo. These in vivo studies may be summarized as follows: Column fractionation had no impact on the precursor B cells or spleen colony-forming cells On the other hand, passage through Ig anti-Ig columns removed mature BM B cells and precursor T cells (17).

Since pre-B cells were not removed by our fractionation procedures, we can conclude that pre-B cells are non-adherent, non-phagocytic cells with little Ig, FcR or C3R on their surface and with no ADCC activity. They would seem to respond to LPS in the same way as mature Ig^+ BM B cells Thus, the LPS responsiveness would seem to be a relatively safe functional marker for the B cell lineage (1,8)

As regards the pre-T cells, the present results are more difficult to evaluate Two findings are of particular importance 1) Mouse bone marrow contains relatively many lymphoid cells with ADCC potential, and these ADCC reactive cells fractionate together with BM Con A responding cells and with pre-T cells, i e they are retained on HSA anti-HSA and Ig anti-Ig columns. 2) the thymus contains cells with ADCC potential (Table 4). These thymus cells also respond vigorously to Con A (Table 3) and are θ positive (unpublished data). Thus, the FcR^+ BM cells with ADCC activity might either be the actual BM pre-T cells or cells which were necessary for the differentiation of BM

pre-T cells into thymic T cells. In both cases, it could be expected that FcR^+ cells with ADCC activity would be found in the thymus. The conception that FcR^+ cells with ADCC activity were the BM pre-T cells is supported by recent reports that T cells might express ADCC activity (7, 11, 12, 19, 20). However, further isolation of and functional studies on the FcR^+ BM cells with ADCC activity are necessary in order to clarify this issue

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THE PREVALENCE AND POSSIBLE SIGNIFICANCE OF IgD GRANULOCYTE-SPECIFIC ANTINUCLEAR ANTIBODIES IN NEUTROPENIC AND NON-NEUTROPENIC CASES OF RHEUMATOID ARTHRITIS

ALLAN WIIK and HENRIK PERMIN

Laboratory of Immunology, University Clinic for Infectious Diseases, Rigshospitalet, Copenhagen, Denmark

Wiik, A & Permin, H The prevalence and possible significance of IgD granulocyte-specific antinuclear antibodies in neutropenic and non-neutropenic cases of rheumatoid arthritis Acta path microbiol scand Sect C, 86 19–22, 1978

Sera from 21 rheumatoid arthritis patients with accompanying neutropenia ($\leqq$2000 neutrophils/µl) and 45 rheumatoid arthritis patients without neutropenia were studied for the occurrence of IgD granulocyte-specific antinuclear antibodies Such antibodies were found in 67 per cent of the neutropenic and 18 per cent of the non-neutropenic cases ($p<0.001$) The titres of IgD granulocyte-specific antinuclear antibodies varied independently of the titres of IgD and complement-fixing granulocyte-specific antinuclear antibodies, but showed some covariation with granulocyte-specific antinuclear antibodies of the IgA and IgM classes most probably reflecting a broad polyclonal antibody response to phagocyte nuclear antigens in the serologically highly active neutropenic cases Gel filtration studies on selected sera containing IgD granulocyte-specific antinuclear antibodies indicated participation of these antibodies in immune complexes It is thus possible that IgD granulocyte-specific antinuclear antibodies may have some significance for the rheumatoid inflammatory processes

Key words Antinuclear antibodies, IgD, rheumatoid arthritis, neutropenia, non-neutropenia

A Wiik, Laboratory of Immunology, Department M 1831-9, Rigshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

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The role of IgD class immunoglobulins in health and disease is not yet fully established It has been postulated that IgD may play a pathophysiological role in certain situations since IgD has been found in the glomerular lesions of various chronic glomerulonephritides (11, 12, 17) and in cutaneous vasculitis (19) and since autoantibodies of the IgD class have been detected (3, 5, 9–11, 13, 16, 18, 24)

The purpose of this study has been to determine the prevalence of granulocyte-specific antinuclear antibodies (GS-ANA) and organ-non-specific ANA (ON-ANA) of the IgD class in sera from rheumatoid arthritis (RA) patients exhibiting varying degrees of serological activity, and to extend previous observations of suchs antibodies in RA (24) Another aim was to establish whether or not such antibodies may participate in circulating immune complexes in RA and thus be suspected of having pathophysiological significance.

MATERIALS AND METHODS

Sera were obtained from 21 neutropenic (neutrophils $\leqq$ 2000/µl) and 45 non-neutropenic cases of definite or classical RA (14) The age and sex distribution of the patients is illustrated in Table 1 The former group represents cases of very pronounced serological activity as has been pointed out in previous publications (1, 2, 15, 20–22, 25) For control purposes we also studied sera from 66 age and sex matched normal donors without any signs of chronic diseases

Sera were studied for presence of IgG, IgM, IgA, IgD and complement-fixing GS-ANA and ON-ANA as previously described (23–25), and positive samples were titrated in two-fold dilutions using phosphate buffered saline pH 7.4 (PBS) as diluent. Fluorescein isothiocyanate labelled IgG fractions of rabbit antisera specific for human γ, μ, and α chains as well as the human β1c component of C3 were obtained from Dako, Copenhagen, and for human δ chains from Behringwerke, Marburg, Germany. The specificity of the anti-Ig conjugates was checked by crossed immunoelectrophoresis and direct immunofluorescence technique on monoclonal bone marrow specimens from patients with myelomas (IgG, IgA, IgD), macroglobulinaemia (IgM) and on a continuously cultured myeloma E cell line (kindly donated by Dr. *K. Nilsson*, Uppsala) (24, 25). Results were read in a Leitz Orthoplan microscope equipped for incident light illumination as already described in detail (23) Rheumatoid factors were demonstrated by the F II latex fixation slide test (Behringwerke, Marburg, Germany)

TABLE 1. *The Age and Sex Distribution of the Groups Studied*

Patient Group:	No ·	Ratio ♀/♂ (%)	Age. (mean, range)
RA with neutropenia	21	76/24	63 0 (42-82)
RA without neutropenia	45	77/23	63 3 (33-80)
Normal donors	66	76/24	63 0 (33-82)

Eleven sera containing IgD GS-ANA were fractionated on a G-200 Sephadex column at pH 7 35 using Tris-HC1 buffer, ι = 0 154 (21). Six of these sera were also fractionated on G-200 Sephadex at pH 4 50 using acetate buffer, ι = 0 154 (21). Immediately after the fractionation all of the latter fractions were neutralized to pH 7 4 by addition of NaOH All fractions were studied undiluted after dialysis against PBS for the presence of IgD ANA

RESULTS

The prevalence of IgD class ANA in the two groups of RA patients is illustrated in Table 2. The clearly higher prevalence in the neutropenic group is due to the occurrence of IgD GS-ANA in most of these sera, whereas IgD ON-ANA was equally rare in both of the patient groups. The difference in IgD GS-ANA frequency is highly significant ($p < 0.001$) (4). Titration of the IgD GS-ANA showed a maximum titre of 32 in both groups, but the geometrical mean titre was 5.9 in the neutropenic group and 1.4 in the non-neutropenic group. All of the normal control sera lacked IgD ANA activity.

TABLE 2. *The Prevalence of IgD GS-ANA*

Patient Group.	Total Prevalence of IgD ANA (%).	Prevalence of IgD GS-ANA (%)
RA with neutropenia	81	67[a]
RA without neutropenia	27	18[a]
Normal donors	0	0

[a] $X^2 = 15.90$, $p < 0\ 001$

To evaluate whether IgD GS-ANA titres showed any correlation to the titres of other Ig classes of GS-ANA, to complement-fixing GS-ANA or to the titres of rheumatoid factors, all titre values were compared by Spearman's rank correlation tests (4) No covariation was seen between IgD GS-ANA and IgG GS-ANA titres, complement-fixing GS-ANA titres or rheumatoid factor titres, whereas some covariation was seen between GS-ANA titres of IgD and IgA ($p < 0.02$) and IgD and IgM ($p < 0\ 01$)

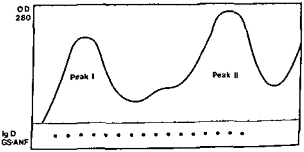


Fig 1a 3 ml of serum from a patient with rheumatoid arthritis and accompanying neutropenia subjected to Sephadex G-200 gel filtration at pH 7 35 in Tris-HCl buffered saline containing 0.02 M sodium azide, ι = 0 154. ● designates IgD GS-ANA activity in the individual fractions obtained Abscissa Fraction no

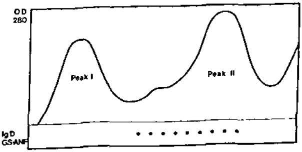


Fig 1b. 3 ml of the same serum subjected to Sephadex G-200 gel filtration at pH 4 50 in acetate buffered saline containing 0 02 M sodium azide, i = 0 154 ● designates IgD GS-ANA activity in the individual fractions obtained after neutralization and dialysis against PBS Abscissa: *Fraction no.*

Fig 1a and 1b depict the gel filtration profiles of a representative serum at pH 7.35 and pH 4.50. At neutral pH IgD GS-ANA is found both in macromolecular and in 7S containing fractions indicating aggregation of some of the IgD antibodies The elimination of macromolecular IgD GS-ANA upon gel filtration at acid pH probably designates immune complex dissociation (6, 21) Ten out of 11 sera fractionated at neutral pH contained macromolecular IgD GS-ANA, while only one out of 6 sera still showed presence of macromolecular IgD GS-ANA after acid fractionation However, all of the sera thus treated still contained 7S IgD GS-ANA.

DISCUSSION

In a previous study from this laboratory, IgD GS-ANA could be demonstrated in 11% of randomly selected GS-ANA positive RA patients (24). The present study shows a very similar prevalence in non-neutropenic cases of RA, however, a significantly higher prevalence is found in neutropenic cases A number of studies have demonstrated pronounced serological activity in this particular group of RA patients which often have high serum titres of rheumatoid factors (2, 15, 25), high titres of IgG and IgM GS-ANA (22, 25) and shows presence of complement-fixing GS-ANA (25), considerable amounts of circulation immune complexes (1, 8, 20, 21), exhibits hypocomplementaemia (8) and often leakage of GS-ANA into the urine probably due to latent immune complex glomerulitis (22). The common demonstration of IgD GS-ANA in these patients most probably designates a massive polyclonal antibody response to nuclear antigens from degrading phagocytes after their involvement in type III reactions in the circulation (1). The covariation between IgD and IgA and IgM GS-ANA titres, respectively, supports this notion of a broad polyclonal response. Unwanted cross-reactivity with other Ig classes of one more of the conjugates used cannot be completely excluded as an explanation of this covariation due to the higher sensitivity of the indirect than the direct immunofluorescence technique used for the specificity controls On the other hand, the existence of a number of sera containing high titres of IgA or IgM GS- ANA in the absence of IgD GS-ANA and vice versa makes the latter explanation less likely.

IgD has been detected in the glomerular deposits in glomerulonephritis and it has been hypothesized that IgD thus migt have some significance for the immune complex mediated tissue damage (11, 12, 17) The present study more directly shows that IgD may be involved in serum aggregates which probably represent immune complexes due to the fact that the aggregates disappear at acid pH (6, 21), though admittedly, such acid dissociability may not be sufficient proof of the immune complex nature of the aggregates (7). The IgD molecules bound to granulocyte nuclei and thus recorded as macromolecular IgD GS-ANA could represent IgD antibodies of other specificities such as antiglobulins as discussed in previous communications dealing with apparently macromolecular IgG and IgA GS-ANA (21, 23). The involvement of IgD in immune complexes in RA is also made probable by the observation that IgD GS-ANA were found i 3 out of 4 cryoprecipitates isolated from sera of Felty patients (*A. Wiik*, unpublished work)

The present study thus establishes the existence of GS-ANA belonging to the IgD class of immunoglobulins, shows the likely independence of this class of GS-ANA from IgG, IgA, IgM and complement-fixing GS-ANA as well as classical rheumatoid factors, and indicates a high prevalence of IgD GS-ANA in neutropenic cases of RA where they seem to participate in circulating immune complexes. The pathogenetic significance of these findings remains to be established.

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IS THE DIABETOGENIC EFFECT OF STREPTOZOTOCIN IN PART THYMUS-DEPENDENT?

KARSTEN BUSCHARD and JØRGEN RYGAARD

Pathological-Anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

Buschard, K & Rygaard, J Is the diabetogenic effect of streptozotocin in part thymus-dependent? Acta path microbiol scand Sect C, 86 23–27, 1978

Following a single injection of 200 mg streptozotocin/kg BW, 25 out of 25 normal mice became diabetic, whereas 12 (= 20%) out of 59 athymic nude mice did *not* develop diabetes.
In a multiple dosage experiment, where the same total dose was given over a 5 days period, 14 normal and 15 athymic nude mice all became diabetic, but nude mice had significantly lower blood glucose values These results support our previous suggestion that a thymus dependent immune reaction is, in part, responsible for the diabetogenic effect of streptozotocin

Key words streptozotocin, diabetes mellitus, thymus, cell-mediated immunity, nude mice

K. Buschard, Pathological-Anatomical Institute, Kommunehospitalet, DK-1399, Copenhagen K, Denmark.

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The induction of diabetes mellitus with streptozotocin in experimental animals, especially mice, is a standard model in the study of diabetes (*Rerup* 1970)

A total single dose of 200 mg streptozotocin/kg Body Weight (BW) invariably induces diabetes mellitus in normal mice (*Rerup & Tarding* 1969) We tried to induce diabetes mellitus in athymic nude mice with the same single dose and found less sensitivity in these mice than in normal mice (*Buschard & Rygaard* 1976) We suggested that the absence of a cell-mediated immune response in nude mice might account for the different outcome.

Recently, *Like & Rossini* (1976) reported a more slowly developing diabetes mellitus in normal mice after administration of 40 mg streptozotocin/kg BW for five days, i e the same total dose as in the single dosage experiments mentioned Islets of Langerhans were infiltrated with lymphocytes. *Like & Rossini* also suggested a cell-mediated immune reaction to be involved

We have compared the reaction of normal mice and athymic nude mice to both single and multiple dosage injections of streptozotocin in order to further elucidate the possible role of thymus dependent immune responses in streptozotocin induced diabetes mellitus.

MATERIALS AND METHODS

Mice

Three groups of mice were studied

1) 8 week old nude mice, mostly males, the outcome of the 10.–11 backcross mating to BALB/c These mice were bred at the Patologisk-anatomisk institut, Kommunehospitalet, Copenhagen (PAI), or purchased from Gl Bomholtgaard, DK-8680 Ry, Denmark (BOM)

2) 8 week old normal BALB/c, mostly males, purchased from BOM.

3) 8 week old NSH mice, all males

The NSH mice have been bred at the Nordic Insulin Laboratory, Gentofte, Denmark.

Mice from PAI and BOM were bred under spf-conditions, NSH mice under conventional conditions. During experiments all mice were kept conventionally, two by two in Makrolon® cages, type II NSH mice were kept at the Nordic Insulin Laboratory, all other mice at PAI as formerly described (*Rygaard* 1973). Autoclaved food pellets and sterile drinking water was supplied *ad lib*

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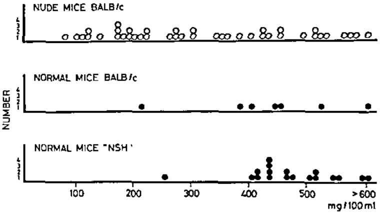


Fig 1 Blood glucose values of mice in single dosage experiments
One circle, open or filled, represents one mouse

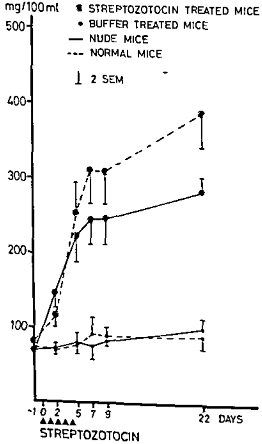


Fig 2 Results of multiple dosage experiments Average blood glucose values of mice tested on the day denoted by the abscissa are given with 2 SEM



Fig 3 Islet of Langerhans of streptozotocin treated normal mouse, day 6 after treatment Slight lymphocytic infiltration is seen. H & E.x 200.

normal BALB/c mice examined by day 6 after multiple dosage treatment was there slight lymphocytic infiltration of islets of Langerhans (Fig. 3)

Streptozotocin

Streptozotocin originates from Upjohn (Kalamazoo, Mich. USA). In single dosage experiments streptozotocin was diluted in saline. To normal mice and nude mice, 200 mg/kg BW was injected in the tail vein within 4 min of preparation. In multiple dosage experiments, streptozotoxin was diluted in citrate buffer, pH 4.2, immediately before intraperitoneal injection. Forty mg/kg BW was given daily for five successive days.

Experimental groups

Streptozotocin treatment was given to 59 nude mice, 8 BALB/c and 18 NSH in *single dosage experiments*, and to 15 nude mice and 14 BALB/c in *multiple dosage experiments*.

Of the latter, 2 and 7, respectively, were females

Controls: in single dosage experiments, using saline for dilution, no buffer controls were injected. Reference blood glucose values were taken from other experiments, and ≧ 200 mg/100 ml was considered diagnostic of diabetes. In multiple dosage experiments, 7 nude and 7 BALB/c mice were injected with citrate buffer; 1 and 4, respectively, were females.

Blood Sampling and Testing

Blood for glucose determinations was collected in 50 µl pipettes from the paraorbital venous plexus of non fasting animals In single dosage experiments blood was taken once a week, in multiple dosage experiments every 2–3 days for the first 9 days, cfr. Fig 1

Blood glucose levels were determined spectrophotometrically following incubation with orthotoluidin reagent.

Observation

Animals were observed daily, and spontaneous death was registered In single dosage experiments, all survivors were used for transplantation experiments (*Buschard & Rygaard* 1976) On day 6 after termination of the multiple dosage scheme, 17 of the mice were sacrificed for histological study 6 streptozotocin treated nude and normal mice, and 3 and 2 buffer controls respectively. Other animals were sacrificed in terminal condition or at the end of experiments

Histology

Tissues were taken and prepared for histological study Following formalin or Bouin fixation, specimens were embedded in paraffin, cut at 5 µm and stained in hematoxylin and eosin and aldehyde fuchsin (*Lillie & Fulmer* 1976)

Statistical Studies

The Mann-Whitney U test was used as significance test

RESULTS

Single Dosage Experiments

Out of the total of 59 streptozotocin treated mice, 18 (= 31%) died within the first week of experiment, before blood samples were taken Results of blood glucose testing in survivors are given in Fig. 1. Diabetic blood glucose values, i e ≧ 200 mg/100 ml, are recorded in the figure when first obtained, within the first or the second week of experiment. Blood glucose values for individual mice not becoming diabetic are all results of second week measurements in order to be sure that such animals are not just slow responders. These mice were followed for several weeks and never developed signs of diabetes.

A total of 29 nude mice (= 49%) became diabetic with average blood glucose values of 395 ± 50 (2 SEM).

Twelve (= 20%) nude mice did not become diabetic after the arbitrary criterion of diabetes applied in this single dose study, chosen as high as blood glucose ≥ 200 mg/100 ml. These animals had an average blood glucose of 152 ± 24.

Out of the 8 normal BALB/c mice, 1 died. The average blood glucose value of the 7 survivors was 442 ± 103, and all were individually diabetic. Likewise, all normal 18 NSH mice became diabetic (459 ± 55). Blood glucose values for both of these groups are given after one week.

Multiple Dosage Experiments

Results of multiple dosage experiments are shown in Fig. 2. Average blood glucose values of mice tested on the day denoted by the abscissa are given with 2 SEM.

It is seen from the figure that blood glucose values are going up already after 2 days out of the 5-day-treatment. This increase is significantly higher (days 2–22) in treated nude and normal mice as compared to buffer controls ($p < 0.01$). If streptozotocin treated nude and normal mice are compared, it will be seen that nude mouse blood glucose values are lower than values in normal mice from day 5. The difference between the levels increases and is statistically significant from day 7 ($p < 0.05$). Blood glucose values of buffer injected controls remain unaffected during the period of the experiment. Female mice of both groups had slightly lower blood glucose values than male, but statistical testing showed no significant difference.

Histology

The histological examination of the pancreas of normal and nude mice showed largely normal findings. Only in 2 out of 6 streptozotocin treated

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DISCUSSION

This study has confirmed our previous finding that nude mice respond weaker to the diabetogenic effect of streptozotocin than do normal mice.

This is true both in single and multiple dosage experiments, using the same total dose of 200 mg/kg BW.

Nude mice are born athymic, thus lacking thymus-dependent immune responses, i.e. the ability to raise a cell-mediated immune response (*Pantelouris* 1968; *Rygaard* 1969, 1973). As normal mice of the background strain show a more pronounced responsiveness to streptozotocin, we have suggested that a cell-mediated reaction is a cofactor in the development of streptozotocin diabetes (*Buschard & Rygaard* 1976). This preliminary finding of a differential response in nude and normal mice in the single dosage model has now been confirmed in the multiple dosage model.

That it is especially the lacking immune response of the nude mouse which seems to determine its lesser response to streptozotocin may be derived from the following preliminary observations (*Buschard* to be published):

Nude BALB/c mice were injected with spleen cells from normal BALB/c mice. This procedure will lead to a total or partial immunological reconstitution (*Kindred & Weiler* 1972). Streptozotocin was given a week later according to the multiple dosage model. The nude mice injected with spleen cells had, both after 2, 5, 7 and 9 days significantly higher blood sugar values than did streptozotocin treated control mice. Apart from the second day (as in the present study) the blood sugar values did not, however, quite reach the level of the corresponding normal mice, but – as already mentioned – the reconstitution may not be complete.

The multiple dosage model was introduced by *Like & Rossini* (1976). They used Charles River CD-1 mice and found a slight delay in the development of diabetes as compared with the single dosage model, in that early signs of diabetes were found only by day 4 vs. day 2. Furthermore, they demonstrated a lymphocytic infiltration of islets of Langerhans, which, they, too, took as an indication of an involvement of the immune system.

In our opinion, the finding by *Volk & Wellmann* (1976) that cortisone pretreatment of mice will lead to a less pronounced diabetogenic effect of streptozotocin in single dosage, can also be interpreted in favour of an involvement of the immune system, supposing that the effect of cortisone is via its immunosuppressive activity.

However, most nude mice did become diabetic in single dosage experiments, and all nude mice became diabetic in the multiple dosage experiments, although to a significantly less degree than normal mice. This suggests that streptozotocin may cause a primary damage to the insulin producing cell, secondarily further accentuated by an immune reaction. It is known that streptozotocin is toxic to the B-cell via a depletion of cellular NAD (*Wyse & Dulin* 1971; *Schein et al.* 1973,) an effect that can be abrogated by pretreatment with nicotinamid (*Schein et al.* 1967).

Like & Rossini suggested the involvement of a cell-mediated immune reaction because of their finding of a lymphocytic infiltrate in the islets of Langerhans. We have not seen similar massive infiltrates in the islets of our mice, normal or nude. *Like & Rossini* used Charles River CD-1 mice, we used BALB/c. This may well explain the differences in histological findings. These authors also found large numbers of C-virus particles in the islets of their diabetic mice. We have found no evidence of C-virus particles in our material. (*Buschard & Rygaard*, unpublished).

The concordant functional test underline, however, that such inflammatory changes are not necessary to bring along the secondary effect that we also suppose to be immunologic on the basis of our previous and present findings.

Whatever the antigen, and whatever immune reaction involved, our findings in nude mice and *Like & Rossini*'s findings in normal mice suggest that the antigen is thymus-dependent.

Present work in our laboratory is aimed at the classification of what type or types of immunological reaction are involved.

K.B. is a research fellow of the *Michaelsen Foundation*. *Jytte Kræmmer* gave skilful technical assistance. The study was supported by the *Danish Medical Research Council* grant nr. 512-5732. Our thanks are due to all mentioned.

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PASSIVE TRANSFER OF VIRUS INDUCED DIABETES MELLITUS WITH SPLEEN CELLS

KARSTEN BUSCHARD

Pathological-Anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

Buschard K. Passive transfer of virus induced diabetes mellitus with spleen cells. Acta path. microbiol. scand Sect. C, 86: 29–32, 1978

The study describes passive transfer of diabetes mellitus by transplantation of spleen cells from donor mice infected with a diabetogenic encephalomyocarditis virus. Inbred normal C57 mice were used as both donors and recipients, and other recipients were C57 athymic, nude mice. Transplants were made in two series after 12 and 22 days duration of infection of the donors, respectively. All recipients became diabetic. The possibility of virus transfer with the spleen cell transplant is discussed, and found to be highly improbable. The fact that passive cellular transfer can be effected points to a decisive significance of the thymus dependent lymphocyte system in the pathogenesis of the primary diabetes.

Key words: Passive cellular transfer, diabetes mellitus, diabetogenic virus, nude mice.

K. Buschard, Pathological-Anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark.

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The following describes transfer of a virus induced diabetes mellitus, confirmed by glucose tolerance tests, by transplantation of spleen cells.

An earlier study (2) implicates the thymus dependent lymphocyte system in the pathogenesis of diabetes induced by encephalomyocarditis virus (EMC). It was concluded that it was a thymus dependent immune response rather than the virus as such that destroyed the insulin producing cells. This pathogenetic postulate led to the following study designed to examine the possibility of passive cellular transfer of a virus induced diabetes.

MATERIALS AND METHODS

Experimental Animals

Donors: twenty six eight week old normal inbred male C57/BL/6/BOM mice served as donors.

Fourteen were injected with the virus, and 12 with the virus free vehicle.

Recipient: 1. thirty six animals of the same age, sex and breed as the donors and 2. thirty two eight week old nude mice (11th backcross of gene transfer to C57/BL/6).

Approximately half of the recipient mice (thirty eight) were transplanted with spleen cells 12 days after the donors were infected, and the remainder after 22 days (see Table 2, which also differentiates between experimental and control animals). All mice were purchased from Gl. Bomholtgaard, Laboratory Animals Breeding and Research Center, DK-8680 Ry, Denmark.

The mice were reared under specified-pathogen-free (spf)-conditions. Over the experimental period the animals were kept in pairs in Makrolon® cages (type II) at the Royal Veterinary and Agricultural University of Copenhagen. Autoclaved feed pellets (Gl. Bomholtgaard) and sterile drinking water was supplied ad lib.

Virus, Virus Assay, and Virus Inoculation

An M-strain of the Encephalomyocarditis virus (EMC), known to be diabetogenic, originating from Craighead's Laboratory (3) was used. A fifth passage batch of virus was titrated in L-cells (established cell line of mouse fibroblastic cells) to a titre of $10^{5.5}$ – $TCID_{50}$/ml. Mice were inoculated intraperitoneally with 0.2 ml of virus suspension containing $10^{4.0}$ $TCID_{50}$. Controls were inoculated with the suspension medium alone.

Spleen Cell Preparation and Inoculation

Immediately on sacrifice of the donor mice, the spleen was removed and fine dealt in BSS with surgical

tweezers. Connective tissue elements were removed, and the remaining cell material was agitated with a pipette to effect cell separation. The test tube was then placed on ice for five minutes, over which period there was sedimentation of the larger particles. The supernatant was then drawn off and diluted to a volume of 10 ml per spleen. The suspension was centrifuged at 200 *G* for 10 minutes at 4° C. The supernatant was discarded, and the cell remnant placed in 1 ml BSS Cell concentration was adjusted to 8×10^7/ml after total counts made after Türk's staining in a counting chamber. A standard transplant of 4×10^7 viable cells in 0.5 ml BSS was injected intraperitoneally into recipients Control recipients received similar transplants from donors injected with virus free vehicle.

Virus Antibodies

All recipients were examined for the presence of neutralizing antibodies to EMC virus three weeks after the spleen cell inoculation. Blood samples were taken from the paraorbital venous sinus with a 50 µl pipette. Serum from the mice was pooled in pairs, and a conventional neutralization test performed of EMC virus cultured in L-cells.

Metabolic Studies

Surviving virus treated mice underwent glucose tolerance tests (GTT) at two and three weeks after virus inoculation Recipient mice were tested at two days and at one, two and three weeks after the spleen cell transplantation The two day test in mice transplanted with cells from donors 12 days after infection was performed in only about half of the animals. Otherwise all surviving animals were tested

After 24 hours fasting, glucose 2 mg/g BW was given intraperitoneally. Blood samples were taken from the paraorbital venous plexus with 25 µl pipettes at time 0, 30 and 60 minutes Blood glucose levels were determined spectrophotometrically by the orthotoluidine method

Statistical Studies

The Mann-Whitney U-test was applied to determine significance

RESULTS

Results of GTT's are shown in Tables 1 and 2. Only the 30 minutes values are recorded. These are the most relevant for the GTT method used (Figs. 1 and 2 show all three time values). Blood sugar values are recorded with 2 SEM. and where there is statistical difference between experimental and control animals, this is denoted. Table 1 concerns donor mice, and it can be seen that the virus infected mice consistently display higher blood sugars than the controls ($p<0.01$), a finding which confirms previous reports (2).

TABLE 1. *Glucose Tolerance Test Results in Donor Mic*

Donor mice	Glucose tolerance test Values at 30 min – mg/100 ml	
	2 weeks	3 weeks
Virus C57	(7) 308 ± 29[a]	(6) 292 ± 47[a]
Control C57	(4) 182 ± 30	(6) 175 ± 20

[a]: $P<0.01$

Average blood glucose values ± 2 SEM.
( ) number of mice in each group

Table 2 concerns recipient mice. The two day study shows no effect in normal mice and moderate effect in the nude mice, but at one week all experimental animals display statistically significantly higher blood sugar values than do the comparable controls. At two weeks the difference is yet more marked, though not in nude mice transplanted from donors after 22 days infection In this group there were only two survivors among the controls, and blood sugars were tending to normalize in the surviving experimental animals. At three weeks, blood sugars in the mice transplanted from

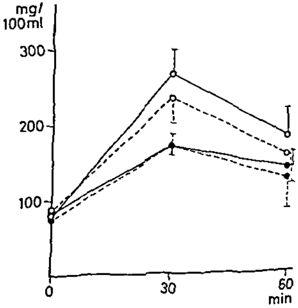


Fig 1. Glucose tolerance test results after two weeks[a] in recipient mice transplanted with spleen cells from donors 12 days after infection

Continuous line = normal mice
Broken line = nude mice
Open circles = recipients of virus lymphocytes
Filled circles = control mice
(*confidence limits 2 SEM*)

[a] most markedly diabetic test results

TABLE 2. *Blood Glucose Values in Normal and Nude Recipient Mice after Spleen Cell Transfer*

Donor	Donors sacrificed on day (transplant day)	Recipient	Glucose tolerance test Values at 30 min – mg/100 ml			
			2 days	1 week	2 weeks	3 weeks
Virus C57	12	C57	(6) 167 ± 14	(12) 225 ± 33[a]	(12) 265 ± 34[b]	(12) 216 ± 29[a]
Control C57	12	C57	(4) 171 ± 20	(9) 173 ± 14	(6) 171 ± 12	(6) 147 ± 28
Virus C57	12	C57 nu/nu	(7) 188 ± 11[a]	(8) 202 ± 29[b]	(7) 235 ± 33[a]	(4) 164 ± 31
Control C57	12	C57 nu/nu	(5) 155 ± 27	(6) 152 ± 16	(5) 171 ± 16	–
Virus C57	22	C57	(8) 172 ± 25	(8) 234 ± 37[a]	(5) 274 ± 80	(5) 298 ± 59[a]
Control C57	22	C57	(7) 159 ± 10	(7) 172 ± 17	(4) 172 ± 38	(4) 168 ± 17
Virus C57	22	C57 nu/nu	(8) 183 ± 16[a]	(8) 211 ± 27[b]	(4) 136 ± 46	(4) 142 ± 33
Control C57	22	C57 nu/nu	(6) 148 ± 23	(6) 150 ± 13	–	–

[a] $P<0.05$
[b] $P<0.01$

Average blood glucose values ± 2 SEM ( ) number of mice in each group

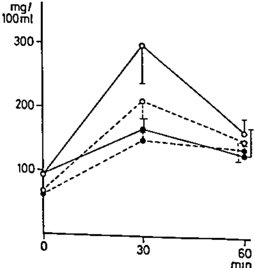


Fig 2 Glucose tolerance test results after three weeks[a] (normal mice) and one week[a] (nude mice) following transplantation of spleen cells from donors 22 days after infection

Continuous line = normal mice
Broken line = nude mice
Open circles = recipients of virus lymphocytes
Filled circles = control mice
(confidence limits 2 SEM)

[a] most markedly [illegible]st results

donors after 12 days infection were falling (the majority (five out of seven) of nude controls had died), but the normal mice transplanted from donors after 22 days infection displayed their highest blood sugar levels Figs. 1 and 2 show complete GTT curves for mice at the time of peak effect. This was at two weeks in both nude and normal mice transplanted from donors after 12 days infection (Fig.1), but at one week (nude mice), and three weeks (normal mice) in the recipients transplanted from donors after 22 days infection. Time O values are approximately the same in both experimental and control animals, but clear differences are apparent at 30 minutes

Studies for neutralizing antibodies were negative in all mice (nude and normal) transplanted from donors after 12 days infection, but weakly positive (titre 0 – 1:10) in all animals (nude and normal) transplanted from donors after 22 days infection.

DISCUSSION

The findings of the study indicate that a diabetes mellitus state primarily induced by a diabetogenic virus can be passively transferred to a recipient animal by transplantation of spleen cells. Presupposing that there is not transfer of virus, the study results as a whole strongly support an earlier

tweezers Connective tissue elements were removed, and the remaining cell material was agitated with a pipette to effect cell separation. The test tube was then placed on ice for five minutes, over which period there was sedimentation of the larger particles. The supernatant was then drawn off and diluted to a volume of 10 ml per spleen. The suspension was centrifuged at 200 G for 10 minutes at 4° C. The supernatant was discarded, and the cell remnant placed in 1 ml BSS. Cell concentration was adjusted to 8×10^7/ml after total counts made after Turk's staining in a counting chamber. A standard transplant of 4×10^7 viable cells in 0.5 ml BSS was injected intraperitoneally into recipients. Control recipients received similar transplants from donors injected with virus free vehicle.

Virus Antibodies

All recipients were examined for the presence of neutralizing antibodies to EMC virus three weeks after the spleen cell inoculation. Blood samples were taken from the paraorbital venous sinus with a 50 µl pipette. Serum from the mice was pooled in pairs, and a conventional neutralization test performed of EMC virus cultured in L-cells

Metabolic Studies

Surviving virus treated mice underwent glucose tolerance tests (GTT) at two and three weeks after virus inoculation Recipient mice were tested at two days and at one, two and three weeks after the spleen cell transplantation. The two day test in mice transplanted with cells from donors 12 days after infection was performed in only about half of the animals. Otherwise all surviving animals were tested.

After 24 hours fasting, glucose 2 mg/g BW was given intraperitoneally. Blood samples were taken from the paraorbital venous plexus with 25 µl pipettes at time 0, 30 and 60 minutes. Blood glucose levels were determined spectrophotometrically by the orthotoluidine method.

Statistical Studies

The Mann-Whitney U-test was applied to determine significance.

RESULTS

Results of GTT's are shown in Tables 1 and 2 Only the 30 minutes values are recorded. These are the most relevant for the GTT method used (Figs. 1 and 2 show all three time values). Blood sugar values are recorded with 2 SEM, and where there is statistical difference between experimental and control animals, this is denoted. Table 1 concerns donor mice, and it can be seen that the virus infected mice consistently display higher blood sugars than the controls ($p<0.01$), a finding which confirms previous reports (2).

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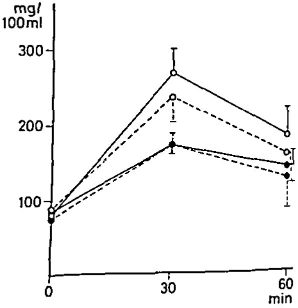


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Acta path microbiol scand Sect. C, 86 33-35, 1978

BRIEF REPORT

AN INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST FOR DETERMINATION OF RUBELLA VIRUS SPECIFIC IgM ANTIBODIES

Elimination of Secondary IgM Rheumatoid Factor Staining after Absorption of Serum IgG with Staphylococcal Protein A

Kjell Skaug and Per Ivar Gaarder

The Microbiological Laboratory, Ullevål Hospital and Department of Immunology, National Institute of Public Health, Oslo, Norway

Skaug, K & Gaarder, P I An indirect immunofluorescent antibody test for determination of rubella virus specific IgM antibodies Elimination of secondary IgM rheumatoid factor staining after absorption of serum IgG with staphylococcal protein A Acta path microbiol scand Sect C, 86 33-35, 1978

Absorption of sera with protein A rich staphylococci eliminated unwanted secondary IgM staining caused by rheumatoid factors in the indirect immunofluorescence test for rubella virus antibodies The absorption did not lower the sensitivity for IgM rubella antibodies as compared with the haemagglutination inhibition test on the IgM serum fractions obtained by ultracentrifugation

Key words Rubella, IgM antibodies, immunofluorescence test, protein A absorption

K Skaug, The Microbiological Laboratory, Ullevål Hospital, Oslo 1, Norway

Received 19 ix 77 Accepted 2 xi 77

The indirect immunofluorescence (IF) technique has been used successfully for the detection of rubella specific serum antibodies In this test the IgM antibodies are far more easily detected in IgG-depleted, IgM containing serum fractions than in whole serum (3,9) In addition, when testing whole serum, IgM rheumatoid factor (RF) may bind to IgG virus antibodies resulting in a false positive IgM antibody test (13, 16, 18) To prevent this unwanted staining by RF in the IF test, pretreatment of sera with aggregated human gammaglobulin (13, 16) or with anti-γ-Fc (5), to remove either the RF or the serum IgG respectively, has been assayed

The elimination of IgG by absorption to protein A of *S aureus* has been used for demonstration of rubella virus specific IgM by the haemagglutination inhibition (HI) test (1, 12, 14) This report describes an IF technique for the detection of rubella specific IgM antibodies, after absorption of serum IgG with staphylococcal protein A This IF technique eliminates the disturbing secondary RF fluorescence

Materials and methods

Sera Thirty serum specimens from patients with a serologically confirmed acute and previous rubella infection, were collected for this study and stored at −20°C for 6–12 months All the sera contained IgG rubella antibodies and 14 of them also IgM antibodies, as detected in the HI test after separation of the IgG and IgM by sucrose gradient centrifugation (19) The sera with rubella IgM antibodies were all nagative in the RF latex test (Latex-RF-reagens, Behringwerke A/G) All the IF tests were performed on coded sera

Forty-five RF positive sera were obtained from adult patients with rheumatoid arthritis The RF titres varied from 32 to 8000, with an almost equal distribution within this range

Absorption of serum IgG by protein A/staphylococci. The IgG antibodies were absorbed from the sera by a protein A rich *S aureus*, Cowan I strain (4, 11) prepared as described by *Jonsson & Kronvall* (10) 0 1 ml of serum was added to 0 57 ml of a 30 per cent (v/v) suspension of staphylococci in phosphate buffered saline (PBS),

postulate that a thymus dependent immune response is responsible for islet B cell damage rather than the virus as such.

An immediate reaction to these conclusions could be that the whole is explained by transfer of active virus together with spleen cells, but this is highly improbable:

a) Recipients of spleen cells from donors sacrificed 12 days after infection displayed nil titres of virus antibodies. Mice primarily infected with the virus display titres at 1:250 after two weeks and 1:1250 after three weeks (*Buschard,* to be published). The antibody trace found in mice transplanted with cells from donors sacrificed 22 days after infection, hardly reflect virus infection, but rather the transfer of B lymphocytes, stimulated in the donor to produce virus antibody. The fact that antibody trace is only found in recipients receiving transplants from donors after 22 days infection can reflect findings from a concurrent study that antibody titre is higher in donors after 22 days infection than after 12 days infection (*Buschard,* to be published).

b) Virus can be cultured from donor mice, but only in very small quantities, from heart and pancreas, organs for which the virus has greatest affinity, as late as the 12th day after infection ($< 10^{-4}$ of that quantity which can be cultured on the 4th day), but after 17 days all cultures are non productive yet more certainly so on the 22nd day (*Buschard,* to be published).

c) Another consideration of weight is the reaction of the nude mouse: the animal displays a diabetic GTT curve after spleen cell transplant, but not after infection with the virus (2). The logical postulate is that the active agent is not viral.

Finally (although not as a real argument in this context) it should be mentioned that passive transfer has been demonstrated in an analogous study of chemically (streptozotocin) induced diabetes mellitus. Spleen cell transplants were performed 12 days after the initiation of streptozotocin treatment of the donors at which time donors were markedly and increasingly diabetic (1). The present study involved transplantation after 12 and 22 days infection of the donors with the virus. Table 2 shows that diabetes was more marked in recipients of cells from the latter donor (22 days infection), which may reflect more marked diabetes in donor animals after the longer period of infection. It is possible though, that the antibodies found in very low titre in recipients of cells from donors after 22 days infection could be adjunctively involved in increasing the degree of the primarily cellular diabetogenesis. This possibility is being investigated.

Other authors (4) have studied passive transfer of allergic adrenalitis and encephalomyelitis in rats effected by lymph node cell transplants. It was found that cells in the recipients collaborated in the destructive process initiated by the donor lymph node cells. This was evidenced by negation of the pathological effects of the donor cells in sublethally irradiated recipients. A similar collaboration mechanism might be involved in the passive transfer of virus induced diabetes.

If so, the nude mouse must be possessed of the mentioned adjunctive cells. These cells cannot be thymus dependent in the athymic nude – and are therefore unlikely to be thymus dependent in normal mice. – But of primary importance in the pathogenesis of both primary and transferred diabetes would seem to be the thymus dependent immune system as suggested in and by this present study and previous viral studies (2).

K. B. is a Research Fellow of the *Michaelsen Foundation*. *Tove Vang* and *Jytte Kræmmer* gave skilful technical assistance.

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cocci on the RF activity, the RF titres were examined both before and after absorption. Of the 45 sera tested, 16 had unchanged titres, while 23 and 6 had a titre reduced by one and two twofold steps, respectively.

Discussion

The rubella specific IgG and IgM serum antibodies are commonly demonstrated by the HI method on sucrose gradient fractions. However, some difficulties have been reported such as tailing of IgG into the IgM fraction (15) and the inadequate removal of non-specific inhibitors (8). The method is rather time-consuming, and an IF test for rapid detection of specific IgM antibodies has been developed.

Several previous reports have shown a high sensitivity of the rubella and other virus IgM fluorescence tests for demonstrating the virus specific IgM antibodies in serum fractions with reduced IgG concentration (3, 9, 17). When whole serum is tested, the IgM antibody staining may be rather weak. This is probably due to competition between the different immunoglobulin classes for the same virus antigenic determinants, and/or that the larger IgM molecules do not reach virus antigens in the cells as efficiently as the smaller IgG molecules. In this investigation, a similar effect was observed. For the five rubella IgM antibody sera titrated in the IF test, somewhat higher IF titres were detected with the absorbed sera. Furthermore, the specific granular fluorescence was much more distinct and easy to read in the IgG absorbed specimens, as these tests were almost free from non-specific staining. Both HI negative and positive rubella IgM sera were examined and when the IF test was performed with absorbed sera, there was complete agreement between the results of the HI and IF tests.

It has been shown that RF positive sera with virus specific IgG antibodies only may give positive IgM IF tests. The RF presumably react with IgG antibodies attached to intracytoplasmic viral antigens in the fixed cells, thus giving non-specific results. This is a serious disadvantage, and different methods for eliminating this disturbing secondary IgM fluorescence have been reported (5, 13, 16). In the present work, all untreated sera that contained both RF and rubella specific IgG antibodies showed a positive IgM IF test, while the RF positive sera without demonstrable rubella IgG antibodies were negative in the IgM test. However, after treatment of the specimens with protein A/staphylococci, the secondary fluorescence was eliminated, with the exception of one sample with high titred RF. This residual fluorescence was, however, clearly also caused by RF, since it was completely removed after absorption with insolubilized IgG.

Staphylococcal protein A reacts with human IgG subclasses IgG_1, IgG_2 and IgG_4, and thus absorption with staphylococci selectively removes these three IgG sub-classes (11). It is of interest that the RF most frequently encountered in sera from patients with rheumatoid arthritis is directed against an antigen, Ga, present on all IgG molecules of these same three sub-classes, while another common RF specificity reacts with Gm(a) antigen of IgG_1 (6). Thus absorption of sera with staphylococci removes the three IgG sub-classes most commonly involved in reactions with rheumatoid factors, thus preventing the interference of RF in the IgM IF test. The protein A/staphylococci absorption of the serum IgG does not particularly affect the RF activity of the serum specimens, since the RF titres were unchanged or only weakly reduced after absorption.

The present IgM IF test is a convenient and reliable test for demonstration of rubella specific IgM antibodies and is well suited for routine and screening serology. It circumvents both the problem of reduced and unwanted secondary IgM staining when testing whole serum. The technique compares adequately with the sensitivity and specificity of the HI method on sucrose gradient fractions.

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pH = 7.4. Immediately after mixing, the bacteria were pelleted by centrifugation, and the supernatant was tested for rubella IgM antibodies by the IF test. To ensure that a sufficient number of bacterial cells was added to the different sera, twice the number of cells needed for absorption of the IgG from a serum pool from several blood donors was used. After this treatment, 80 per cent of the IgM and only 5 per cent of the IgG still remained in the supernatant of samples from the serum pool, as determined by radial immunodiffusion.

Absorption of rheumatoid factor. 0.1 ml aliquots of some af the RF positive sera were mixed with 20 mg heat denaturated (64°C for 20 minutes), glutaraldehyde insolubilized human IgG (2) suspended in 0.4 ml PBS. The solutions were mixed continuously for 3 hours at room temperature and then overnight at 4°C. The samples were centrifuged at 4°C and the supernatants, corresponding to a five-fold dilution of the serum, were tested by the IF technique.

The indirect immunofluorescence test was performed on glass slides, using smears of BHK-21 cells infected with a high multiplicity of rubella virus, strain RA-27/3, fixed in acetone for 10 minutes at 4°C. The serum dilutions were incubated with the cell smears in a moist chamber at 37°C for 1 hour for determination of IgG antibodies and for 3 hours for determination of IgM antibodies. They were then washed and incubated for 45 minutes with suitable dilutions of fluorescein isothiocyanate conjugated rabbit anti-human IgG and anti-human IgM antibodies (Dakopatts A/S), respectively. The conjugates were tested for specificity as described by *Skaug & Tjötta* (17). Only a typical yellowish green granular cytoplasmic fluorescence was regarded as positive. For screening, the sera were tested in ten-fold dilution for detection of IgG antibodies and in five-fold dilution for detection of IgM antibodies. The glass slides with the fixed cells could be stored at 4°C for several months without appreciable loss in their specific reactivity in the IF test.

The haemagglutination inhibition test was carried out according to the method described by *Halonen et al.* (7). The IgG and IgM antibodies were separated by ultracentrifugation of serum on a 10–40 per cent sucrose gradient at 100,000 × *g* for 24 hours

Rheumatoid factor test Our modification of the Waaler-Rose test was performed in microtitre plates with U-wells, each well containing 50μl of serum diluted in 0 15 per cent bovine serum albumin in PBS (BSA/PBS) and 50 μl of a 0.3 per cent suspension in BSA/PBS of human 0 erythrocytes sensitized with approximately ¼ of an agglutinating amount of rabbit IgG antibodies to human erythrocytes Sedimentation patterns were read after incubation at 4°C overnight

Results

Sensitivity and specificity of the rubella IgM IF test To compare the IF and the HI tests for detecting the rubella specific IgM antibodies, the IF test was performed with whole serum and with serum absorbed with staphylococci and the HI test with sucrose gradient fractions Five acute phase sera from rubella patients were titrated

TABLE 1. *Rubella Virus IgM Antibody Titres in Haemagglutination Inhibition (HI) and Immunofluorescence (IF) Tests Performed with Unabsorbed Serum and Serum Absorbed with Protein A/Staphylococci*

Serum No.	IgM titre: HI test*	IgM titre: IF test, Unabsorbed serum	IgM titre: IF test, Absorbed serum
1	32	10	20
2	16	20	40
3	16	10	10
4	32	5	20
5	32	5	20

* The titre of the IgM sucrose fraction showing the strongest reactivity.

(Table 1). For the five sera investigated, the IF titres were somewhat higher with absorbed serum specimens than with whole serum. When testing unabsorbed sera, several specimens showed a non-specific fluorescence which was difficult to distinguish from the specific IgM rubella staining

The 30 coded sera, 14 of which showed rubella IgM antibodies detected in the HI test after sucrose gradient centrifugation, were examined by this IgM IF test The sera with specific IgM antibodies showed a positive IgM IF test and the specimens with rubella IgG antibodies only, showed a negative IgM IF test

Interference of RF with the IgM IF test The 45 RF positive serum specimens were examined unabsorbed and after absorption with staphylococci in the IF test When tested unabsorbed, 40 RF sera with a positive rubella IgG IF test showed a positive IgM IF test After absorption all these sera, except one, were negative in the rubella IgM IF test The remaining five sera which were negative in the rubella IgG IF test, were also negative in the IgM IF test both before and after absorption of IgG

After absorption, the serum which was weakly positive in the IgM IF test had 5 per cent IgG left, but still had an RF titre which was reduced from 8000 to 4000 only. This serum and 14 other RF positive sera were treated with heat denaturated insolubilized human IgG One absorption reduced the RF titres by from 16 to more than 256 times The one serum with a positive IgM IF test after absorption with staphylococci then showed a negative IgM IF test However, one of the remaining sera which had a RF titre reduced from 4000 to 32, was still positive. After two additional absorptions with insolubilized IgG, this specimen showed an RF titre below 16 and a negative IgM IF test Generally, the IgM IF tests on untreated RF positive sera showed such intense fluorescence in some samples that a granular pattern was difficult to observe.

To check the effect of the absorption with staphylo-

Acta path microbiol scand Sect C, 86 37–40, 1978.

BRIEF REPORT

IMMUNE COMPLEXES IN THE SPUTUM OF PATIENTS WITH CYSTIC FIBROSIS SUFFERING FROM CHRONIC *PSEUDOMONAS AERUGINOSA* LUNG INFECTION

P O Schiøtz[1], *H Nielsen*[2], *N. Høiby*[3], *Grace Glikmann*[2] *and S -E Svehag*[2]

[1]) The Paediatric Clinic TG and Statens Seruminstitut, Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
[2]) University Institute of Medical Microbiology, Odense, Denmark
[3]) Statens Seruminstitut, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen, Denmark

Schiøtz, P O, Nielsen, H, Høiby, N, Glikmann, G & Svehag, S -E. Immune complexes in the sputum of patients with cystic fibrosis suffering from chronic *Pseudomonas aeruginosa* lung infection Acta path. microbiol scand Sect C, *86* 37–40, 1978

12 cystic fibrosis (CF) patients chronically infected with mucoid *P aeruginosa* and presenting multiple precipitins in serum against this bacterium and 12 patients without *P aeruginosa* infection were examined for occurrence of soluble immune complexes in their sputum sol phase by a complement consumption assay and a solid phase rheumatoid factor binding assay The correlation between the results obtained in the two assays was significant ($r = 0.625$, $p < 0.01$) The patients chronically infected with *P aeruginosa* showed a significantly ($p < 0.01$) higher frequency of immune complex activity in their sputum sol phase, as compared to the patients without *P aeruginosa* lung infection These findings point to the possibility that chronic lung infection with mucoid *P aeruginosa* in CF may be an immune complex disease

Key words Cystic fibrosis, immune complexes *Pseudomonas aeruginosa*

P O Schiøtz Paediatric Department TG, Rigshospitalet, Tagensvej 18, DK-2200 Copenhagen N, Denmark

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In vivo formation of soluble immune complexes in serum have recently been demonstrated in patients with cystic fibrosis (CF) (19) These findings were clearly associated with patients suffering from chronic *P aeruginosa* lung infections who are known to have a pronounced humoral immune response and a poor prognosis (10, 18)

The origin of the antigen(s) participating in the immune complex formation has not been established However immune complex formation in the lungs between *P aeruginosa* antigens and the large number of specific precipitins formed to these antigens have been proposed earlier as a possible explanation (8, 11, 18)

The purpose of the present study therefore has been to investigate the occurrence of soluble immune complexes in the sputum sol phase in CF patients with chronic *P aeruginosa* infection compared to CF patients without *P aeruginosa* infection

Patients and Methods

Patients Twentyfour CF patients were included in the study All patients had a typical history of CF and marked elevated sweat electrolytes in repeated tests (5) Routine studies of the lung function showed a reduced vital capacity and peak expiratory flow rate as compared with normal values (22)

(i) One group consisted of 12 CF patients (5 females, 7 males, median age 16 5 years, range 8–29) suffering from chronic infection with mucoid strains of *P. aeruginosa* (CF+P) and exhibiting more than 10 different precipitins in serum against water soluble antigens from these bacteria The patients have been followed as previously described (8,9) Mean duration of the *P aeruginosa* respiratory tract infection was 4 5 years (range 1–7)

(ii) The other group consisted of 12 CF patients (6 females, 6 males, median age 13 years, range 7–21) without *P. aeruginosa* infection (CF–P) and with none or

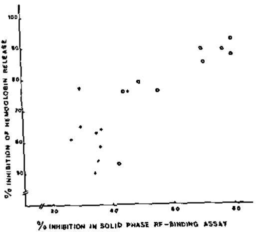


Fig 2 Correlation between the complement consumption test and the rheumatoid factor binding assay 18 cystic fibrosis patients were examined for immune complex activity in the sol phase of sputum by the two assays ($r = 0.625$, $p < 0.01$).
0 indicates patients chronically infected with *P aeruginosa*
+ indicates patients without *P aeruginosa* infection

both of the hemoglobin release in the CC test and the specific binding of ΔIgG in the RF-BA than sputum from patients in the CF-P group, $p < 0.01$ (Figs 1 and 2) These findings indicate the presence of soluble immune complexes in the sputum of CF patients chronically infected with mucoid *P aeruginosa* A Spearman's analysis showed a positive correlation between the data obtained by the two assays ($r = 0.625$, $p < 0.01$) Only 18 patients were tested by RF-BA because of lacking sputum material

Discussion

Patients with CF suffer from recurrent and chronic bacterial lung infection (9) Chronic lung infection caused by *P aeruginosa* presents the most severe problem today (15) The pronounced antibody response against the infecting bacteria notably *P aeruginosa* (14) and the persistence of the bacterial antigens during the chronic infection provide suitable conditions for the formation of immune complexes in the lungs of CF patients Our study indicates that in vivo formation of soluble immune complexes does take place in the lung in CF patients, and predominantly in patients suffering from chronic infections with mucoid *P aeruginosa* These patients have a multitude of precipitating *P aeruginosa* antibodies in their sera The presence of these precipitating antibodies in serum has been shown to correlate to the presence of antibodies in sputum of CF patients (18), but there was not enough sputum from our patients to permit a simultaneous study of immune complexes and precipitins However, it seems reasonable to assume that the immune complex activity we have demonstrated in the sputum of the CF + P patients is due to immune complexes formed by *P. aeruginosa* antigens and specific *P. aeruginosa* precipitins Furthermore, the circulating immune complexes previously demonstrated in serum of the CF + P patients (19) may well originate from the lungs. Definite conclusions do however require identification of the specific antigen(s) and/or antibodies in the immune complexes in sputum and serum which is attempted in studies under way.

The CC test most effectively detects immune complexes formed in slight to moderate antibody excess (17) This may explain why immune complex activity was not detected in one of our CF + P patients, who presented a very pronounced antibody response against *P aeruginosa* (40 precipitins)

Our results also point to the possibility that immune complexes can be formed in the lungs of CF patients during infections caused by other microorganisms than *P aeruginosa* , as the sputum sol phase of 2 CF−P patients suffering from *S aureus* and probably viral respiratory infection, respectively, also exhibited strong inhibition of hemoglobin release suggesting immune complex activity. The complement consumption observed with sputum from the CF patient suffering from *S aureus* infection could possibly also be due to complement activation induced by complexes of IgG with protein A of *S aureus* (20)

The indicated presence of complement activating immune complexes in sputum sol phase and their close correlation to chronic infection with mucoid *P aeruginosa* as well as the occurrence of many *P aeruginosa* precipitins in the patient's sera may explain why these infections are associated with a poor prognosis in CF patients (15) Complement activation followed by attraction of neutrophils, release of lysosomal enzymes from the neutrophils with tissue damaging effect and activation of the coagulation system, the kinin-generating system and the fibrinolytic system through the Hagemann factor are the mechanisms of immune complex induced tissue damage (2, 4, 7, 16) Hypersensitivity reactions to *P aeruginosa* antigens have also been demonstrated in vivo in CF + P patients, supporting the concept of immune tissue damage in these patients (13)

According to our results, chronic lung infection with mucoid *P aeruginosa* in CF patients, like several other chronic infections (23) may turn out to be an immune complex disease But a study of the pathogenic importance of the immune complexes found in sputum of CF patients is necessary to provide the full evidence The possible therapeutical consequences of our findings is currently investigated

This work was supported by the *Family Hede Nielsen Foundation*, the *Danish Medical Research Council* (project no 512-7193 and 512-6623) and the *National Danish Association against Cystic Fibrosis* We thank *Anni Bethien, Lise Schrøder, Elsebeth Thomsen* and *Ellinor Ward Petersen* for skilful technical assistance

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few serum precipitins against *P. aeruginosa* antigens. In 9 of the CF-P patients *S aureus, H. influenzae, E. coli* or *K. ozaenae* were isolated from the respiratory tract during this study.

Sputum. A 3-h-sample of sputum was collected at 4°C from each patient between 8 a m. and 11 a.m as described previously (1, 18) Each sample was subjected to bacteriological examination and the origin of the specimens from the lower respiratory tract was confirmed by studying the epithelial cells present (9, 18) Sol phase of sputum was obtained by ultracentrifugation at 120.000 x g (maximum value) at 4°C for 4 h and stored in small aliquots at − 80°C (18).

Crossed immunoelectrophoresis. The occurrence of circulating precipitating antibodies in serum against *P. aeruginosa* were investigated by means of crossed immunoelectrophoresis (microtechnique) as described previously (10) A close correlation between the presence of precipitating antibodies in serum and sputum sol phase has been observed (18)

Complement consumption assay The complement consumption test (CC) was performed as previously described (17) All sputum samples were tested within the same day and again two days later. The mean value of these determinations is expressed as per cent inhibition of hemoglobin release produced by the samples from each patient A standard preparation of *P aeruginosa* antigens, which produced 60 immunoprecipitates when tested by crossed immunoelectrophoresis against a rabbit antiserum to *P aeruginosa* (12), was included in the CC test as antigen control. The introduction of this antigen preparation into the test system had no significant influence on the degree of hemoglobin release as compared to when the buffer control alone was used The antigen preparation was tested in concentrations equal to or exceeding those found in sputum from CF patients (18) The analytical error of the CC assay was determined through a period of one month in 10 different tests using a standard monomer human IgG preparation (8µg/ml) The total coefficient of variation was 3 5%

Preparation of ¹²⁵I-labelled, aggregated IgG Aggregated IgG (△IgG) was prepared by heating 20 mg human IgG (Cohn's fraction II Kabi, Stockholm, Sweden) in 1 ml NaCl-Tween-BSA solution at 63°C for 12 min Two ml of the aggregated material was filtered on a Sephadex G 200 column (2 6 × 100 cm, Pharmacia, Uppsala, Sweden) equilibrated with 0 01 M Tris-HCl buffer containing 0 5 M NaCl and 0 02% NaN_3, pH 8 0 The column was run at 4°C and the flow rate was 5 ml/hour The fractions eluted in the first peak (≥ 22S) were pooled and concentrated to 10 mg/ml Iodination of the heat aggregated material was performed by the lactoperoxidase method (21) The labelled preparation (10 µCi/µg protein) was separated from free iodide on a small Sephadex G-25 column and stored at −60°C.

Solid phase rheumatoid factor binding assay A polyclonal rheumatoid factor (RF) IgM preparation, isolated by column chromatography, and a RF negative IgM similarly isolated from normal human serum was used for coating plastic tubes (6). The latex globulin agglutination titer of the RF IgM preparation was 1280, (0.5 mg protein/ml). The tubes were incubated with 5 µg of each IgM preparation in 1 ml PBS for 18 hours at room temperature, thereafter the tubes were washed 3 times with a 0.10 M NaCl solution containing 0 2% BSA and 1% Tween 20 The test sample (final dilution 1:800) and 50 ng of ^{125}I △IgG in 1 ml NaCl-Tween-BSA solution was added to each tube and the tubes were incubated for 3 hours at room temperature. Control tubes received only the indicator ^{125}I△IgG preparation in NaCl-Tween-BSA. All tubes were washed 3 times with PBS and bound counts were determined in a Biospan scintillation detector (Nuclear Chicago) Results were expressed as per cent inhibition of specific binding of ^{125}I△IgG to RF IgM (counts bound to RF IgM − counts bound to control IgM). In routine testing of serum samples for immune complex activity the total coefficient of variation for the solid phase rheumatoid factor binding assay (RF-BA) was 12%.

Statistical methods. The Mann-Whitney test and Spearman's test were employed and a significance level of 5% (double tailed test) was chosen (3)

Results

The sputum sol phase of the patients in the CF+P group caused a significantly higher degree of inhibition

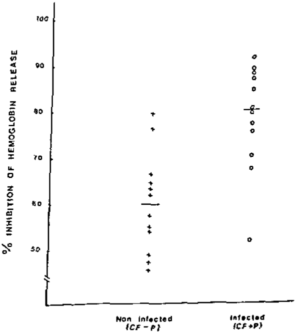


Fig 1. Anticomplementary activity in the sol phase of sputum from patients with cystic fibrosis determined by a complement consumption test Degree of anticomplementary activity in sol phase of sputum from patients with cystic fibrosis with (CF + P) and without (CF−P) *P aeruginosa* infection, p<0 01.

Acta path microbiol scand Sect. C, 86 41–43, 1978.

BRIEF REPORT

LANCEFIELD EXTRACT OF GROUP A STREPTOCOCCI TYPE 15 ACTS LIKE AN ANTI-HUMAN IgG WITH RESTRICTED SPECIFICITY

Claës Schalén, Poul Christensen and R. Grubb

Institute of Medical Microbiology, University of Lund, Lund, Sweden

Schalen, C, Christensen P & Grubb, R Lancefield extract of group A streptococci type 15 acts like an anti-human IgG with restricted specificity Acta path microbiol scand Sect. C, 86: 41–43, 1978.

Lancefield extract of streptococci group A type 15 precipitates certain human sera and agglutinates red cells coated with some IgG anti-Rh antibodies The frequency of precipitation varies between populations The serum component in the precipitate is IgG The results indicate that an iso-allotypic marker is detected by the extract

Key words Lancefield extract, streptococci, anti-human IgG, iso-allotypes, Gm markers, human Ig

C Schalen, Institute of Medical Microbiology, University of Lund, Solvegatan 23, S-223 62 Lund, Sweden

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In 1959, *Wood & Schramm* (12) described a capillary precipitation reaction between Lancefield-extract of group A, type 15 streptococci and all of the 137 human normal sera tested In some respects the streptococcal factor responsible for the precipitation resembled the M protein, while the serum factor participating was not characterized

Many streptococci including group A, type 15, have receptors for human IgG (2, 4, 8) Extracts *ad modum* Lancefield are known to contain these receptors (3) It was thought worth while to find out whether this Lancefield extract had in fact properties of an anti-human gammaglobulin

Materials and Methods

Streptococcal extract Group A streptococci, type 15 (EF 1949), were cultured on 4 × 5 l Todd Hewitt broth, containing 0 5% glucose, at 37°C for 6–8 hours using magnetic stirrers, each 5 l was inoculated with 0 3 l over night culture of type 15 pH was adjusted to 7 0 every 30–60 min with 2 N NaOH during growth The bacteria were harvested in a continuous action rotor in a Hi-spin 21 centrifuge (MSE, Sussex, England) at 9,000 rev/min Forty ml wet volume of bacteria washed in 0 9% saline was suspended in 100 ml saline and the pH lowered to 2 0 by HCl The suspension was heated in a boiling waterbath for 10 min, after which the pH was adjusted to 7 0 with NaOH, the bacteria were centrifuged and the supernatant was removed and kept at 4°–8°C. The following day a cloudy precipitate was removed by centrifugation at 3,000 *g* The final volume of this extract, used in all experiments, was 150 ml and the protein content, determined as described (10), 2 mg/ml Many of the experiments were repeated several times with minor lots of Lancefield extracts of cultures obtained on separate occasions

Human sera Some of the sera had been stored for more than 10 years at −20°C. All sera were heated at 56°C for 30 min before use The IgG, IgA and IgM content of some sera was determined by the electroimmunoassay (9)

Separation procedures. Human commercial IgG and antisera Sera were separated by means of a Sepharose 4B protein A column from Pharmacia (7) A column of human commercial IgG-Sepharose 4B was used, as earlier described (1) All fractions obtained from the columns were concentrated to the volumes of the specimens applied

Commercial human IgG was purchased from Kabi, Stockholm, Sweden, and rabbit antisera to IgA, IgG and IgM from Dakopatts, Copenhagen, Denmark. The anti-IgG3 was a gift from Dr *A Grubb*, Malmo

Tube and gel precipitation Gel precipitation tests were performed as earlier described (11) The precipitation lines between M 15 extract and human sera were visible after 1–2 days

In tube precipitation experiments, 1 ml of serum was mixed with 1 ml of M 15 extract and 4 ml of 0 9 per cent

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Much evidence, viz. the existence of precipitating and non-precipitating sera, the ethnic group differences, the differential action of the extract on a number of anti-Rh coats, the variation in the capacity of individual sera to inhibit the haemagglutination observed, suggests that the streptococcal extract interacts with some IgG molecules but not with others As for the nature of this restriction, an important clue is that reacting IgG molecules were shown to be present not only in the IgG3 subclass but also in at least one of the other subclasses. All known IgG allotypic markers are confined to one subclass (see *Grubb* (5)) This observation alone indicates that the restriction is due to a marker with the character of an iso-allotype The results of the haemagglutination inhibition tests also lend support to this interpretation

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saline added. After 2 hours at 37°C and 3 days at 4°–8°C the precipitate was spun down at 8,000 g for 1 hour and washed once. 0.2 ml PBS was afterwards added. The precipitate was dissolved by incubation at 37°C with 20µl trypsin (Sigma, St. Louis, M.O., USA) 0.6 mg/ml (the M 15 precipitating factor is trypsin sensitive, see *Wood & Schramm* (12)). Trypsinization was stopped by 20 µl soy bean inhibitor (Sigma, St. Louis, M.O., USA) 5 mg/ml. Controls included 1) only serum or only M 15 extract, trypsinized as indicated above, and 2) only trypsin and inhibitor.

Agglutination and agglutination inhibition tests with cells coated with incomplete anti-Rh were performed as in Gm-typing procedures (5) using plastic trays as described (6). The anti-Rh's used included the well known Ripley serum, which was a gift from Dr. *Marion Waller*, Richmond

Results

Gel Precipitation with Human Sera from Various Populations

The Lancefield extract precipitated human sera as follows:

East Greenlanders	3 out of 33	or	9 per cent
Laplanders	27 out of 36	or	75 per cent
South Koreans	25 out of 25	or	100 per cent
Swedes	32 out of 39	or	82 per cent.

Precipitation was usually obtained also with serum diluted 1·4 A few sera gave 2 lines. All sera classified as precipitated gave a line which fused with that of a control serum

Characterization of the Precipitated Component of Human Serum

All of 21 sera chosen for testing because they contained <7 mg IgG per ml failed to give a line in gel precipitation tests Four of 8 sera containing >7 mg IgG but less than 0 7 mg IgM per ml were precipitated, however, and the same was true for 2 out of 5 with a level of IgA below 0.7 mg/ml

In diffusion in gel experiments the precipitation line formed between anti-IgG3 and serum fused with the line obtained between the same serum and the type 15 Lancefield extract Our anti-total-IgG gave broad, indistinct precipitates making the result of the Ouchterlony comparative analysis less clearcut

Precipitates in tubes were obtained from 8 »positive« human sera The precipitates were dissolved as described in »Methods« All 8 preparations were precipitated by anti-human IgG and anti-human IgG3, but not by anti-human IgA or anti-IgM in gel precipitation

The column containing staphylococcal protein A coupled to Sepharose used is known to retain IgG of subclasses 1, 2 and 4 and to let IgG3 run through The fraction eluted and the fraction retained were both precipitated by the M 15 extract The precipitates showed Ouchterlony identity reactions with each other and with the line obtained with unseparated serum

Commercial human IgG KABI was precipitated by the extract but only at Ig concentrations of at least 25 mg per ml This IgG is derived mainly from retroplacental blood and this prompted us to investigate the precipitating capacity of sera from pregnant women. It was found that 9 out of 45 sera from pregnant women (32nd–40th week of pregnancy) precipitated. This figure was significantly lower than that found in non-pregnant Swedes (see above). Further observations related to this finding will be published separately. Unless otherwise stated, no sera from pregnant women were included in the material forming the basis of this communication

Agglutination of Red Cells Coated with Incomplete Anti-Rh and Results of Agglutination-Inhibition Tests

Lancefield extracts diluted several thousand times agglutinated group ORh + red cells coated with anti-Rh Ripley diluted 1:10 at coating. Uncoated red cells were not agglutinated. Trypsin-treated extracts did not agglutinate the coated red cells, neither did extracts which had passed an IgG coated Sepharose column The agglutination by extracts diluted to contain 8–16 agglutinating doses was inhibited by KABI gammaglobulin in a concentration of less than 0 01 per cent.

In preliminary tests we assessed the ability of the extract to agglutinate samples of erythrocytes coated by 12 other incomplete anti-Rh which were of approximately the same titer as judged by Coombs' test using rabbit antihuman-gammaglobulin Agglutination was obtained with 7 of the coated cell samples. As expected, the titer was not as high as with the Ripley coated cells. It was noteworthy that some high-titered anti-Rh did not coat for agglutination by the Lancefield extract Individual sera were tested for their capacity to inhibit agglutination of red cells coated with anti-Rh Ripley as well as with an anti-Rh HUN found useful for typing IgG3 Gm markers Most sera were inhibitory at a dilution of at least 1:25 A small percentage of sera were markedly less inhibitory and quite a few sera were in an intermediate range Sera from pregnant women were inhibitory to the same extent as other categories of sera Nine pairs of mother-newborn sera were examined and the two sera of each pair were equally inhibitory

Discussion

The precipitates observed might be considered to consist of M-protein type 15 and homologous antibodies Such an interpretation is in accordance with the observation that the reacting component of serum is IgG and could, perhaps, also account for the varying incidence of precipitation in the populations studied This interpretation is less likely for the simple reason that type 15 infections are uncommon and, secondly, also because it is known that human antistreptococcal antibodies rarely precipitate extract of streptococcal cells (12) Such interpretation fails to account for the observed agglutination of red cells coated with IgG anti-Rh-antibodies An alternate explanation is that this Lancefield extract interacts with human Ig irrespective of combining sites for streptococci or streptococcal products In our opinion, the data presented make it difficult to avoid this conclusion. We thus ascribe the precipitation and the agglutination of anti-Rh-coated red cells to the content of Ig Fc receptors in the streptococcal extract

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TOXICITY STUDIES WITH HUMAN LEUKOCYTE INTERFERON IN NEWBORN RABBITS

LIISA PYHÄLÄ, EIJA RIMAILA-PÄRNÄNEN, RIITTA KEKOMÄKI, ELLA KOSTIAINEN and KARI CANTELL

Central Public Health Laboratory, College of Veterinary Medicine and Finnish Red Cross Blood Transfusion Service, Helsinki, Finland

Pyhala, L., Rimaila-Parnanen, E., Kekomaki, R., Kostiainen, E. & Cantell, K.. Toxicity studies with human leukocyte interferon in newborn rabbits Acta path microbiol. scand Sect. C, 86: 45–51, 1978

Daily subcutaneous injections of 5 to 10 million units of partially purified human leukocyte interferon were given to newborn rabbits for 2 weeks or 1 month The control groups received mock interferon, saline or nothing The interferon treatment had no overt effect on the development of the animals during the period of treatment. The rabbits treated with interferon had leukocytosis, splenomegaly and prolonged postnatal extramedullary hematopoiesis in the liver and spleen. Certain immune responses were also demonstrated in the rabbits treated with interferon and mock interferon preparations Platelet counts and the serum-ASAT, -ALAT, -LD and alkaline phosphatase values were normal

Key words Human leukocyte interferon, toxicity, rabbit, platelets, hematopoiesis

L. Pyhala, Central Public Health Laboratory, Helsinki 28, Finland

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Partially purified preparations of human leukocyte interferon are being used for systemic treatment of patients with various diseases in a number of current clinical trials (2) In general, intramuscular or subcutaneous injections of about 10^5 units per kg are tolerated well and cause no irreversible toxic effects (7,14,22,23,24) The observed side-effects, which include fever, local pain and mild allergic reactions, can be reduced or eliminated by further purification of the interferon preparations (24, *Merigan* personal communication) However, even the most highly purified preparations as yet available can cause transient thrombocytopenia and leukocytopenia (7, *Merigan* personal communication). Moreover, infants with cytomegalovirus infections treated with interferon have shown slower weight gain and a transient elevation of serum aspartate aminotransferase (1) These effects may well be due to interferon itself, because interferon is known to inhibit cell division in vitro (12,20) Thrombocytopenia may also result from circulating immune complexes unrelated to platelets (11)

Gresser and his co-workers have recently reported observations on the toxicity of potent mouse interferon preparations to newborn mice (8,9). Daily treatment with about 10^8 units per kg for 1–2 weeks from birth could cause lethal liver damage (9) or progressive glomerulonephritis (8). These findings prompted us to make similar studies with human leukocyte interferon in newborn rabbits. Human leukocyte interferon expresses its antiviral activity nearly as well in rabbit as in human cells (15)

MATERIALS AND METHODS

Interferon Human leukocyte interferon was produced and partially purified as described previously (3). Four batches of partially purified interferon (P-IF) were used Three of them contained 10 million units per ml and the fourth 20 million units per ml The specific activities were 2×10^5, 7×10^5, 1×10^6 and 1.2×10^6 units per mg protein

Mock interferon This was prepared in the same way as normal interferon, except that normal chick allantoic fluid was added instead of Sendai virus derived from

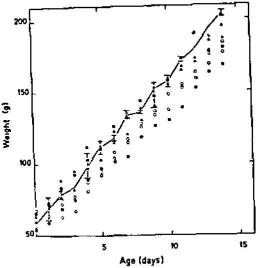


Fig 2 The weight gain of the newborn rabbits in experiment 2 Each rabbit received daily injections of 10 million units of P-IF (●) mock P-IF (○) or saline (▲) on days 0 to 13 The symbols represent individual rabbits The line is drawn through the mean weights of two uninjected rabbits The vertical bars indicate the weight ranges

P-IF, mock P-IF, saline and uninjected groups in either experiments. In fact the inter-individual differences were smaller than those within 5 further uninjected control litters each consisting of 8 young (not shown in the figure)

Interferon Levels in the Sera

In both experiments the interferon levels in the sera were assayed 24 hours after the last injection. In experiment 1, both interferon rabbits had 3500 units per ml of serum In experiment 2, the corresponding values were 11 000 and 2000. In a control experiment two rabbits (aged 30 days and weighing 460 g and 530 g like the rabbits at the end of experiment 1) were injected subcutaneously with 5 million units of P-IF for the first time After 24 hours the serum interferon levels were 35 and 60 units per ml These values agree well with those of our earlier pharmacokinetic studies (4) These findings indicate that the daily subcutaneous administration of 10^8 interferon units per kg results in a cumulative increase of circulating interferon

Blood Cell Counts

It can be seen from Table 1 that intensive treatment with P-IF in experiment 2 had no effect on the platelet count, but it caused moderate leukocytosis

TABLE 1. *Thrombocyte and Leukocyte Counts in the Blood of the Rabbits in Experiment 2 at the Age of 14 Days*

Treatment	Thrombocytes $\times 10^9/l$	Leukocytes $\times 10^9/l$
P-IF	484, 513*	4.1, 4 1
Mock P-IF	586, 637	1.7, 1.7
Saline	436, 550	1.4, 1.5
None	455, 538	2.7, 1.9

*Individual rabbits

Antibodies and Soluble Immune Complexes

Sera from three out of four rabbits treated with P-IF enhanced the binding of IpA by the test platelets (Table 2). When tested in PIA, the same sera showed direct platelet-aggregating activity. This is taken as evidence of antibodies reactive with platelets. In density gradient centrifugation this activity sedimented mainly at the same rate as the 7 S marker, and minimal activity was found in the 19 S region. The sera of the rabbits treated with mock P-IF also showed some activity against platelet reactive antibodies.

The sera from the rabbits treated with P-IF or mock P-IF (Table 2) did not show any HI antibodies against Sendai virus when diluted 1.12.

Serum Enzyme Activities

In all groups of rabbits in both experiments the values for serum-ASAT, -ALAT, -LD and alkaline phosphatase were essentially the same (Table 3). Nor did the sera show any differences in the LDH isoenzyme pattern.

TABLE 2 *Binding of 125-labeled Protein A by Human Platelets Pretreated with the Sera of the Rabbits in the Different Experimental Groups Total Counts Added per Test Tube 8606 cps*

Treatment	Counts per second	
	Exp 1 (day 31)	Exp 2 (day 14)
P-IF	882,310*	237, 96
Mock P-IF		139,150
Saline	44, 69	55, 72
None	79, 44	82, 76
	93, 74	
Platelet control	37	
Normal rabbit serum	97	

*Individual rabbits

chick embryos. After incubation, the cells were disintegrated in a Waring blendor and the normal amount of Sendai virus was added. The crude mock interferon was concentrated 1000-fold and partially purified like the P-IF preparations. The partially purified mock interferon (mock P-IF) contained 17 mg protein per ml. The P-IF preparation used in the same experiment contained 16.4 mg protein per ml.

Interferon assay. Threefold serial dilutions of interferon were titrated by the reduction of VSV plaques on U cells (21). All units are expressed in terms of the international reference preparation 69/19 (National Institute for Medical Research, Mill Hill, London).

Experiments in newborn rabbits. Rabbits of the albino breed were maintained in the Central Public Health Laboratory (Helsinki) under conventional conditions. The young were reared with their dams during the experiments. In the first experiment two young from a litter of 8 rabbits received daily injections of 5 million units of P-IF from birth to the age of 30 days. Two other young were given saline injections at the same time. The remaining 4 young received no injections. In experiment 2 a litter of 8 young was divided into four groups, each 2 animals, as follows: P-IF, mock P-IF, saline and no injections. The daily interferon dose was 10 million units, and the injections were administered on days 0 to 13. All injections were given subcutaneously in a volume of 0.5 ml. The rabbits were weighed daily. Blood samples were collected from the marginal ear veins or by cardiac puncture 24 hours after the last injection.

Blood cell counts. The leukocytes and platelets were counted in a Burger Turk Chamber.

Assays for serum enzymes. Serum-ASAT, -ALAT, -LD and alkaline phosphatase were determined as described previously (6) The LDH isoenzyme was determined according to the Dade Iso-Form procedure (Dade, Miami, Florida) on Microzone cellulose acetate membranes (Gelman, Ann Arbor, Michigan)

Assays for antibodies and soluble immune complexes. The iodinated protein A (IpA) technique (10) and the platelet aggregation sedimentation pattern (PIA) test, kindly performed by Professor *K Penttinen* (17) were used to determine platelet-reactive antibodies or soluble immune complexes in sera The activity found was analyzed further by density gradient centrifugation of the sera (18).

Sera from the rabbits treated with P-IF or mock P-IF were studied for hemagglutination-inhibiting (HI) antibodies (5) to Sendai virus Nonspecific inhibitors were removed with cholera filtrate (Philips-Duphar, B V, Holland) The antigen used was sonicated allantoic fluid from embryonated hen's eggs infected with Sendai virus Fowl erythrocytes were permitted to sediment at 37° C.

Autopsy and histology The animals were killed by dislocation of the neck on day 31 in experiment 1 and on day 14 in experiment 2 The animals were autopsied and light microscopy was used to examine representative specimens from heart kidney, liver, spleen, lymph nodes, thymus, thyroid, adrenals, brain, lung, small and large intestine, ovary or testes, and skeletal muscle The specimens were fixed in 10 per cent formaldehyde or Carnoy, and embedded in paraffin. The histologic sections were stained with hematoxylin and eosin, van Gieson, periodic acid Schiff (PAS) and frozen sections with Scharlach Rot.

In experiment 1 liver specimens were taken for electron microscopy, kindly performed by Dr. *Aili Oksanen*. The specimens were fixed in glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Epon 812. Sections cut at 700–900 Å were lightly stained with uranyl acetate in distilled water and with lead citrate, and examined in a Philips 300 or a Philips 200 electron microscope.

RESULTS

Weight Gains

The growth rates of the rabbits in the two experiments are shown in Figs. 1 and 2. There were no essential differences in the weight gains between

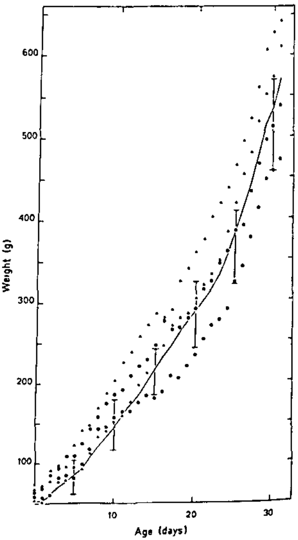


Fig. 1. The weight gain of the newborn rabbits in experiment 1. Each rabbit received daily injections of 5 million units of P-IF (●) or saline (▲) on days 0 to 30 The symbols represent individual rabbits. The line is drawn through the mean weights of four uninjected rabbits. The vertical bars indicate the weight ranges

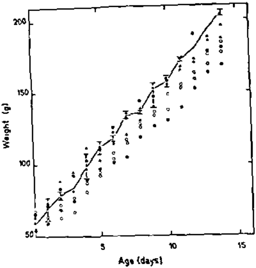


Fig 2 The weight gain of the newborn rabbits in experiment 2 Each rabbit received daily injections of 10 million units of P-IF (●) mock P-IF (○) or saline (▲) on days 0 to 13 The symbols represent individual rabbits The line is drawn through the mean weights of two uninjected rabbits The vertical bars indicate the weight ranges

P-IF, mock P-IF, saline and uninjected groups in either experiments. In fact the inter-individual differences were smaller than those within 5 further uninjected control litters each consisting of 8 young (not shown in the figure)

Interferon Levels in the Sera

In both experiments the interferon levels in the sera were assayed 24 hours after the last injection In experiment 1, both interferon rabbits had 3500 units per ml of serum. In experiment 2, the corresponding values were 11000 and 2000 In a control experiment two rabbits (aged 30 days and weighing 460 g and 530 g like the rabbits at the end of experiment 1) were injected subcutaneously with 5 million units of P-IF for the first time After 24 hours the serum interferon levels were 35 and 60 units per ml These values agree well with those of our earlier pharmacokinetic studies (4). These findings indicate that the daily subcutaneous administration of 10^8 interferon units per kg results in a cumulative increase of circulating interferon.

Blood Cell Counts

It can be seen from Table 1 that intensive treatment with P-IF in experiment 2 had no effect on the platelet count, but it caused moderate leukocytosis

TABLE 1. *Thrombocyte and Leukocyte Counts in the Blood of the Rabbits in Experiment 2 at the Age of 14 Days*

Treatment	Thrombocytes $\times 10^9/l$	Leukocytes $\times 10^9/l$
P-IF	484, 513*	4 1, 4.1
Mock P-IF	586, 637	1.7, 1.7
Saline	436, 550	1.4, 1.5
None	455, 538	2.7, 1.9

*Individual rabbits

Antibodies and Soluble Immune Complexes

Sera from three out of four rabbits treated with P-IF enhanced the binding of IpA by the test platelets (Table 2) When tested in PIA, the same sera showed direct platelet-aggregating activity. This is taken as evidence of antibodies reactive with platelets In density gradient centrifugation this activity sedimented mainly at the same rate as the 7 S marker, and minimal activity was found in the 19 S region. The sera of the rabbits treated with mock P-IF also showed some activity against platelet reactive antibodies.

The sera from the rabbits treated with P-IF or mock P-IF (Table 2) did not show any HI antibodies against Sendai virus when diluted 1:12.

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In all groups of rabbits in both experiments the values for serum-ASAT, -ALAT, -LD and alkaline phosphatase were essentially the same (Table 3). Nor did the sera show any differences in the LDH isoenzyme pattern

TABLE 2. *Binding of 125-labeled Protein A by Human Platelets Pretreated with the Sera of the Rabbits in the Different Experimental Groups Total Counts Added per Test Tube 8606 cps*

Treatment	Counts per second	
	Exp. 1 (day 31)	Exp 2 (day 14)
P-IF	882,310*	237, 96
Mock P-IF		139,150
Saline	44, 69	55, 72
None	79, 44	82, 76
	93, 74	
Platelet control	37	
Normal rabbit serum	97	

*Individual rabbits

chick embryos. After incubation, the cells were disintegrated in a Waring blendor and the normal amount of Sendai virus was added. The crude mock interferon was concentrated 1000-fold and partially purified like the P-IF preparations. The partially purified mock interferon (mock P-IF) contained 17 mg protein per ml The P-IF preparation used in the same experiment contained 16.4 mg protein per ml.

Interferon assay. Threefold serial dilutions of interferon were titrated by the reduction of VSV plaques on U cells (21). All units are expressed in terms of the international reference preparation 69/19 (National Institute for Medical Research, Mill Hill, London).

Experiments in newborn rabbits. Rabbits of the albino breed were maintained in the Central Public Health Laboratory (Helsinki) under conventional conditions. The young were reared with their dams during the experiments. In the first experiment two young from a litter of 8 rabbits received daily injections of 5 million units of P-IF from birth to the age of 30 days Two other young were given saline injections at the same time. The remaining 4 young received no injections. In experiment 2 a litter of 8 young was divided into four groups, each 2 animals, as follows: P-IF, mock P-IF, saline and no injections The daily interferon dose was 10 million units, and the injections were administered on days 0 to 13. All injections were given subcutaneously in a volume of 0.5 ml. The rabbits were weighed daily. Blood samples were collected from the marginal ear veins or by cardiac puncture 24 hours after the last injection

Blood cell counts. The leukocytes and platelets were counted in a Burger Turk Chamber.

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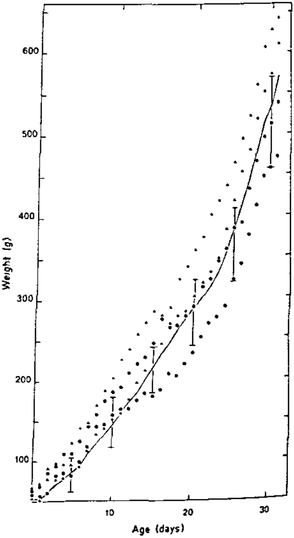


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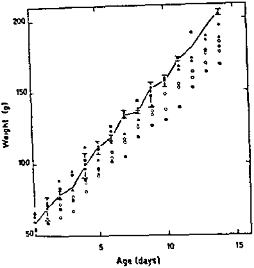


Fig 2 The weight gain of the newborn rabbits in experiment 2 Each rabbit received daily injections of 10 million units of P-IF (●) mock P-IF (○) or saline (▲) on days 0 to 13 The symbols represent individual rabbits The line is drawn through the mean weights of two uninjected rabbits The vertical bars indicate the weight ranges

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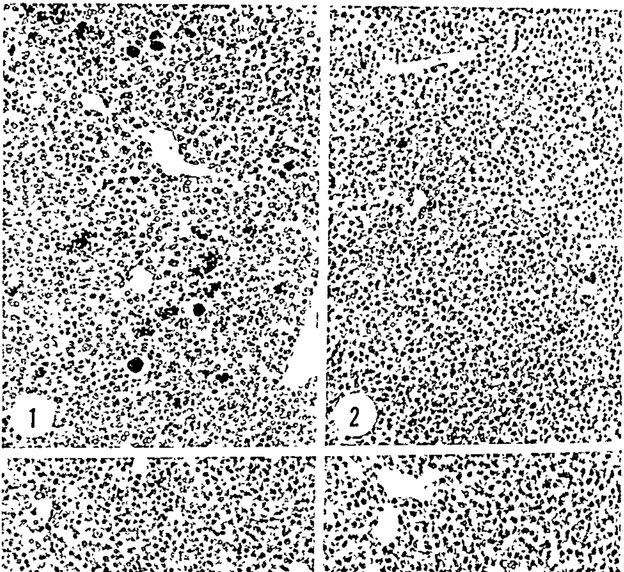

1
2
3
4

Fig 3 A typical histological picture of the rabbits' livers in experiment 2. Hematoxylin-eosin staining. × 150.
Groups 1 P-IF. Massive extramedullary hematopoiesis, many megakaryocytes and immature blood cells.
2 Mock P-IF. Moderate extramedullary hematopoiesis, a few megakaryocytes and immature blood cells
3 Saline No extramedullary hematopoiesis
4 None No extramedullary hematopoiesis

Autopsy and Histological Findings

At autopsy, the main macroscopic finding was a distinct enlargement of the spleen of all the rabbits which had received P-IF. In both experiments the spleens in the P-IF groups weighed two or three times as much as those in the control groups. Mock P-IF did not cause splenomegaly.

The principal histological finding was the prolongation of active extramedullary hematopoiesis at the age of 2 weeks in the livers (Fig. 3) and spleens of the rabbits receiving P-IF and mock P-IF Extramedullary hematopoiesis was most prominent in the P-IF group, and was moderate in the mock P-IF group, but only a few megakaryocytes and immature blood cells were found in the rabbits receiving saline or nothing.

The normal course of disappearance of postnatal extramedullary hematopoiesis in the rabbit strain used in the present studies was elucidated by histological examinations of the livers and spleens of normal rabbits at the ages of 1 day, and 1, 2, 3, and 4 weeks. Each age group consisted of 2 to 4 rabbits In the 1-week-old control rabbits extramedullary hematopoiesis was similar to that in the 2-week-old P-IF rabbits in experiment 2. In the 2-week-old control rabbits, extramedullary hematopoiesis was similar to that in the 2-week-old mock P-IF rabbits. In the control rabbits extramedullary hematopoiesis had totally disappeared by the age of 3 to 4 weeks. This was also the case in all the groups of experiment 1 at the age of 31 days. The course of extramedullary hematopoiesis of control rabbits described above agrees with earlier findings (13).

The specific stains did not reveal any differences in hepatic glycogen and fat content between the groups, nor did electron microscopy of the livers in experiment 1 reveal any cytological abnormalities.

DISCUSSION

At the start of the present experiments the dose of interferon was of the order of 10^5 units per kg. As the rabbits gained weight the dose per kg gradually decreased. On a weight basis the present dosage was as high as that used by *Gresser and his co-workers* in their studies in mice (8,9). We saw no signs of liver or kidney damage. The discrepancy between our findings and those of *Gresser et al.* may have been due to differences in the interferon preparations used or to differences between rabbits and mice. It is possible that the human leukocyte interferon does not express all its biological activities in the different cells and tissues of newborn rabbits, although it has been shown to exert antiviral activity in some rabbit cells (15)

Human leukocyte interferon can cause transient thrombocytopenia in adult human beings (7, *Merigan:* personal communication), but such an

TABLE 3 *Enzyme Values in Sera of Rabbits in the Different Experimental Groups*

Exp	Treatment	Day	ASAT	ALAT	Units/l LD	Alkaline phosphatase
1	Interferon P-IF	24	22,47*	38,67	499,1136	484, 671
	Interferon P-IF	31	34,34		991,1049	465, 503
	Saline	24	13,57	41,62	326,1114	568, 645
	Saline	31	23,61		839, 839	426, 529
	None	24	15,17	34,39	463, 463	632, 658
			20,21	41,44	478, 499	658, 671
	None	31	40,46		644, 666	387, 426
			47,48		854, 877	542, 619
2	Interferon P-IF	14	38,52	19,27	1112,1638	671, 800
	Mock P-IF	14	76,80	23,25	1568,1954	658, 878
	Saline	14	29,36	16,20	948,1030	968,1097
	None	14	30,43	15,20	942, 995	839, 865

*Individual rabbits

effect was not seen in infants (1) or newborn rabbits (present study) during interferon treatment. At the age of 2 weeks, extramedullary hematopoiesis was more prominent in the rabbits treated with P-IF than in the various controls. The absence of thrombocytopenia and leukocytopenia in the young rabbits treated with P-IF may perhaps be due to the prolonged postnatal extramedullary hematopoiesis during the first weeks of life.

Leukocytosis and splenomegaly appeared to be characteristic features in rabbits treated with P-IF. The levels of platelet-associated IgG and serum immune complexes were highest in the rabbits treated with P-IF, but were also elevated in those treated with mock P-IF. These findings reflect the immunogenic stimulation of the young rabbits by the human leukocyte interferon preparations. The antibody-forming capacity in rabbits develops during the first weeks of life (19). In adult rabbits the human leukocyte interferon is an efficient immunogen (16). It was not possible to assay the interferon-neutralizing activity in the sera of the rabbits treated with P-IF because of the very high levels of circulating interferon. Human leukocyte interferon itself has not proved to be immunogenic in human patients, although treatment with interferon preparations may immunize patients against the antigens of Sendai virus and chick allantoic fluid (23). Such immunizations could be avoided with more highly purified interferon preparations, although so far they have not caused adverse clinical effects.

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SPONTANEOUS LYMPHOKINE PRODUCTION BY HUMAN B LYMPHOCYTES

H ARVILOMMI[a], LIISA RÄSÄNEN[b], AINO LAATIKAINEN[c] and ELIISA KARHUMÄKI[b]

[a] Public Health Laboratory, SF – 40620 Jyväskyla 62, [b] Institute of Biomedical Sciences, University of Tampere, SF – 33101 Tampere 10, [c] Department of Cell Biology, University of Jyvaskylä, SF – 40100 Jyvaskyla 10, Finland

Arvilommi, H , Rasanen, L , Laatikainen, A & Karhumaki, E Spontaneous lymphokine production by human B lymphocytes Acta path microbiol scand Sect C, *86*. 53–57, 1978.

When human blood lymphocytes are cultured *in vitro* without any intended stimulus, they produce activities in the supernatant resembling lymphokine This phenomenon was further investigated in the present study, where it has been demonstrated by physicochemical characterization and inhibition experiments that leukocyte migration inhibitory activity in the supernatants is due to leukocyte inhibitory factor (LIF). When T and B lymphocytes were purified by carbonyl iron and SRBC-rosette sedimentation, only B cells produced LIF and leukocyte chemotactic lymphokine(s) in subsequent cultures B cells elaborated lymphokines without the help of T cells the need for co-operation of monocytes was also unlikely T cells were inactive even when reconstituted with monocytes and platelets

Key words B lymphocytes, lymphokines, LIF; leukocyte chemotactic factor.

H Arvilommi, Public Health Laboratory, SF – 40620 Jyvaskyla 62, Finland

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Mechanism of lymphocyte activation have been the subject of intensive research in recent years. This topic has also been extensively reviewed (*Oppenheim & Rosenstreich* 1976, *Wedner & Parker* 1976) The fundamental changes in the lymphocyte after activation include the elaboration of soluble substances called lymphokines, the synthesis of antibodies and transformation into blasts. These events are triggered by a number of stimuli such as antigens, mitogens or various other agents

In our studies of lymphokine synthesis after reactions on the lymphocyte membrane, we encountered a phenomenon in which lymphocytes in culture seemed to produce lymphokine-like migration inhibitory substance without any intended stimulus at all (*Arvilommi & Räsänen* 1975) The preceding paper extended these observations to the chemotactic factor which was also demonstrated in unstimulated culture supernatants In this paper we show that these lymphokines are derived from B lymphocytes

MATERIALS AND METHODS

Separation and Purification of T and B Cells

Mononuclear cells from healthy individuals were obtained by Ficoll-Isopaque centrifugation of heparinized blood.

T and B cells were purified as follows. Mononuclear cells were first incubated with 200 mg carbonyl iron (Fluka AG, Buchs, Switzerland) in Hanks' balanced salt solution (HBSS) supplement with 10% foetal bovine serum (Flow Laboratories, Irvine, Scotland) for 45 min at 37° C Free iron and the phagocytic cells were removed with a magnet. 2×10^7 lymphocytes, 0 5 ml foetal bovine serum (absorbed twice with SRBC), 0 5 ml HBSS, and 1 0 ml 16% SRBC were mixed in 17×100 polystyrene tubes (Falcon, Oxnard, CA , U S A) and centrifuged for 5 min at 200 *g*, followed by incubation for 30 min in ice SRBC were previously treated with neuraminidase (Vibrio comma neuraminidase, Behringwerke, Marburg, Germany) by incubating 20% SRBC in HBSS at37° C for 30 min with 50 U/ml neuraminidase After three washings the cells were ready for use in rosette sedimentation. Rosetted cells were taken from the ice bath and left for 10 min at room temperature, then

TABLE 1. *Results of Cell Separation*

	Unseparated	T Cells	B Cells
SRBC-Rosetting Cells	69.7 (58–76)[a]	95.9 (94–98)	2.6 (1–5)
SMIg-Positive Cells	19.3 (16–23)	2.1 (<0.5–4)	77.3 (71–86)
Monocytes	11.1 (5–25)	<0.5	3 0 (1–4)
Total	100 2 (98–105)	98 0 (95–99)	82.7 (76–93)
Living Cells	98.1 (96–99)	97.0 (95–98)	97.4 (96–99)

[a] Mean % and range

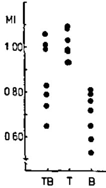


Fig 1 Leukocyte migration inhibitory activity in culture supernatants of unseparated (TB), T, and B cells

proved to be chemotactic possibly because of the lot of horse serum used However, as shown in Fig 2, this background activity was diluted off in higher dilutions, revealing the chemotactic lymphokine in supernatants of unseparated and B cells. Similar results were obtained in experiments where no such chemotactic medium was used. It was possible that these activities were produced not by B cells, but by contamonating platelets which were enriched in the B cell population in the separation experiments. In

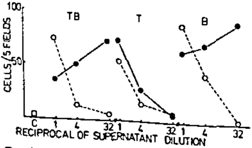


Fig 2 Leukocyte chemotactic activity in culture supernatants of unseparated (TB), T, and B cells ●—37° C supernatants, O- -4° C supernatants C □ medium control

control experiments, platelets were cultured alone or together with T cells and monocytes, but no LIF was found (Table 2). These results also show that T cells remained inactive even when reconstituted with monocytes an platelets.

A crucial point is whether the lymphokine-like activities found in the supernatants can, in fact, be attributed to factors elaborated by lymphocytes. This and previous studies show that these activities were not produced when cell metabolism was blocked by cold or puromycin. In order to obtain

TABLE 2. *Absence of LIF in supernatants of Platelets or T Cells Reconstituted with Monocytes and Platelets*

Exp No	Platelets	T Cells + MON + P
1	1.06[a]	0 98
2	1.02	0 98
3	0.98	1.04
4	1 02	0.97
5	1.04	1 00
6	1.05	1.00
Mean	1 03	1.00

[a] MI

TABLE 3 *Migration Inhibitory Activity in Pooled Fractions of Sephadex G-100 Chromatography*

Exp No	Pooled Fractions I	II	III	IV
1	0.99[a]	0 68	1.02	0 99
2	1.06	0 57	0.93	0 95
3	0 99	0 66	1 00	1.03
Mean	1.01	0 64	0 98	0 99

[a] MI

the cells were resuspended gently and layered on Ficoll-Isopaque 14% Ficoll was used in preparing these Ficoll-Isopaque solutions (*Parish & Hayward* 1974) The tubes were centrifuged for 20 min at 1000 g, and B rich cells were collected from the interphase and subjected to another rosetting – Ficoll-Isopaque cycle. The cell-SRBC pellet was suspended in 1 ml HBSS and a hypotonic shock was given with 3 ml water for 10 s to lyse the SRBC. The non-separated mononuclear cells, T and B cells were washed three times with HBSS and cultured for three days at 37° C or 4° C in MEM for suspension cultures (Flow Laboratories) containing 10% horse serum (Flow Laboratories)

Cell Markers

Monocytes were identified by non-specific esterase activity according to *Yam et al* (1971). SRBC rosette formation was used for identification of T celles. Briefly, 10^6 lymphocytes in 0.5 ml HBSS, 0 1 ml 4% SRBC and 0.6 ml 12% Ficoll (*Pattengale & Reichelderfer* 1975) were centrifuged for 5 min at 200 g, followed by incubation in ice for 30 min.

B cells and Fc receptor bearing cells were identified by readily detectable surface membrane immunoglobulin (SMIg) 10^6 cells were spun down and 50 µl of 1.8 dilution FITC-conjugated anti-immunoglobulin (IgG + IgA + IgM, Beringwerke) was added to the cell pellet After incubation in ice for 30 min the cells were washed three times with phosphate buffered saline (PBS) containing 0.1% sodium azide. A drop of 50% glycerol in PBS was pipetted onto the cell pellet and the suspension transferred to a microscope slide for counting in Leitz Orthoplan microscope using Ploem illumination

Separation of Monocytes and Platelets

Mononuclear cells isolated by Ficoll-Isopaque centrifugation were incubated for 30 min in plastic petri dishes in HBSS supplemented with 10% foetal bovine serum The plates were irrigated with HBSS and shaken to remove non-adherent cells. Adherent cells were detached with a rubber policeman and washed three times with HBSS These cells consisted of 94% monocytes

When mononuclear cells are collected from the interphase of the Ficoll-Isopaque run, suspended in HBSS and spun down, the supernatant contains platelets in abundance. These platelets were collected by centrifugation of the supernatant at 800 g for 10 min, washed three times and used at a concentration of 6×10^7 platelets/ml

Lymphokine Assays

Chemotactic activity of the supernatants was assayed as described previously (*Arvilommi et al.* 1978) Migration inhibitory activity was tested by the leukocyte migration in the agarose test (*Clausen* 1971, *Rasänen & Arvilommi* 1977) with minor modifications. Instead of leukocytes, granulocytes were used After sedimentation, the leukocyte-rich plasma was centrifuged on Ficoll-Isopaque. Cells at the bottom were 98% pure granulocytes. These were washed three times before use as indicator cells in the assay The agarose plates were buffered with 5% CO_2 – $NaHCO_3$ Wells 2.3 mm in diameter were punched in the gel and 5 µl culture supernatant containing 10^6 granulolytes was pipetted into each well. The supernatants were tested with four to six replicate determinations. The area of the well was excluded from the final area of migration.

Characterization of Leukocyte Inhibitory Factor

For gel filtration chromatography, cells were cultured in serumfree MEM for suspension cultures in 17 × 100 Falcon tubes at a concentration of 4×10^6 cells/ ml for two days. Supernatants (30–50 ml) were dialyzed overnight against distilled water and lyophilized The powder was dissolved in 2 ml gel filtration buffer and clarified by centrifugation for 10 min at 1000 g A 1.6 × 85 cm column of Sephadex G-100 was equilibrated with a volatile buffer (0.05M ammonium bicarbonate, acetic acid, pH 7.2) and calibrated with rabbit serum albumin (Sigma, St Louis, Mo., U.S.A , mol.wt. 68000 daltons) And α-chymotrypsinogen-A (Sigma, mol wt 23000 daltons). the eluate was pooled into four fractions Fraction I contained the void volume and material eluting before the albumin marker and fraction II molecules eluting with albumin. The fraction between the albumin and chymotrypsinogen markers was referred to as fractionIII, and fraction IV contained material eluting with the chymotrypsinogen marker. The fractions were lyophilized and dissolved in 1/20 of the original volume of MEM for suspension cultures supplemented with 10%horse serum In inhibition experiments, L-fucose and N-acetyl-D-glucosamine (Sigma) were used, as described by *Rocklin* (1976)

The thermostability of active fractions was studied by incubating them for 30 min in 56° C or 80° C waterbath before the assay.

RESULTS

First we attempted to characterize the cells responsible for spontaneous lymphokine synthesis by separating mononuclear cells into B and T cell populations by removal of carbonyl iron fed monocytes and by SRBC-rosette sedimentation, following which the cells were cultured at 37° and 4° C. These cell populations were tested for B and T cell markers, and the proportion of monocytes and dead cells was determined (Table 1)

Tests for LIF carried out on culture supernatants of these cell populations revealed that unseparated cells elaborated LIF in four out of seven cases, T cells never, and B cells always (Fig. 1). There seems to be no correlation between the number of contaminating monocytes and the production of LIF Leukocyte chemotactic activity was found constantly in the supernatants of unseparated cells and B cells, but T cells again remained inactive. One of the seven experiments performed is given in Fig. 2. In this series of experiments the culture medium

It remains to be investigated whether spontaneous lymphokine synthesis and the background in lymphocyte transformation reflect the same activation processes.

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TABLE 4. *Effect of Heat and Monosaccharides on Leukocyte Inhibitory Activity*

Exp. No.	Control	56°C	80°C	*Control*	N-Acetyl-D-Glucosamine	L-Fucose
1	0.68[a]	0.82	0.97	0 78	0.99	0.80
2	0.57	0.69	1.00	0.66	1.00	0.62
3	0.66	0.75	1.00	0.70	0.91	0 65
Mean	0.64	0.75	0.99	0.71	0.99	0 69

[a] MI

more evidence to support the idea that we were dealing with lymphokines we tried to characterize our LIF candidate physicochemically and performed inhibition studies. In Sephadex G-100 filtration experiments the migration inhibitory activity eluted in fraction II, i.e. in the albumin region suggesting a similar molecular weight of the factor (Table 3). It was partially inactivated by heating at 56° C, and the activity was lost completely at 80° C (Table 4). Furthermore, it was inhibited by N-acetyl-D-glucosamine, whereas α-L-fucose remained ineffective as inhibitor (Table 4).

DISCUSSION

Our earlier papers have shown that human lymphocytes release lymphokine-like activities in *in vitro* cultures apparently without any stimulus Quite recently a confirmative observation was reported by *Anders & Natvig* (1976), who found migration inhibitory activity in their control supernatants in studies of virus-induced cell-mediated immunity. In this investigation, we demonstrated that B lymphocytes are the cells responsible for this spontaneous lymphokine production. The fact that it is lymphokines which are formed is confirmed by the present physicochemical evidence and inhibition experiments. These prove that it is a question of LIF, a lymphokine characterized earlier by others (*Rocklin* 1975, *Lomnitzer et al* 1975, *Rocklin* 1976). As lymphokine synthesis represents one of the consequences of cell activation, it is of considerable interest to try to ascertain why it occurs apparently without any stimulus at all. It is possible, of course, that some peripheral blood lymphocytes are always activated *in vivo* because of the immunological reactions in progress, and that these cells are producing lymphokines *in vitro*. This cannot be excluded in the present study, but one would expect that also T cells would be activated *in vivo* and could be shown to release lymphokines. This was not the case. The fact that T cells were able to react after the separation steps used was supported by the finding that they responded to various mitogens (*Räsänen*, unpublished observations).

Another cause for activation might be any of the steps in the isolation or separation of lymphocytes or in the ingredients in the culture medium. For instance, when B cells were separated on a nylon wool column or after Fc- or C3-rosette formation, they were found to release chemotactic and mitogenic lymphokines (*Mackler et al.* 1974, *Wahl et al.* 1974). Also foetal calf serum present in culture medium may be mitogenic for some lymphocytes (*Andersson & Melchers* 1974). We used Ficoll-Isopaque sedimentation in our isolation and separation processes, and have found previously that the result is the same if sedimentation and carbonyl iron are used instead (*Arvilommi & Räsänen* 1975) Lymphokines were also produced spontaneously in serum-free culture media or media supplemented with heterologous or autologous sera as shown previously. Negative exclusion was used in separating B cells and therefore no interactions in the B cell membrane should have occurred. If the stimulus is hidden in *in vitro* techniques, it remains to be identified If this should be the case, this observation bears on in vitro studies on lymphocyte activation. The methods we used are those commonly applied, and as regards the investigations on B cell specific activating agents, these findings might make interpretation more complicated.

It is of interest that in studies on lymphocyte transformation with separated B and T cells, the background incorporation of tritiated thymidine is higher in B than in T cells (*Littman et al.* 1976, Majuri, unpublished observations). It has been shown that lymphocyte transformation and lymphokine synthesis can occur independently (*Dimitriu et al.* 1974, *Mackler et al.* 1974, *Wahl et al* 1974)

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LEUKOCYTE CHEMOTACTIC ACTIVITY IN CULTURES OF UNSTIMULATED HUMAN LYMPHOCYTES

H ARVILOMMI, AINO LAATIKAINEN and LIISA RÄSÄNEN

Public Health Laboratory, SF-40620 Jyvaskyla 62, Department of Cell Biology, University of Jyvaskyla, SF-40100 Jyvaskyla 10, and Institute of Biomedical Sciences, University of Tampere, SF-33101 Tampere 10, Finland

Arvilommi, H, Laatikainen, A & Rasanen, L. Leukocyte chemotactic activity in cultures of unstimulated human lymphocytes Acta path microbiol scand, Sect. C, 86 59–62, 1978.

We have shown earlier that unstimulated human lymphocytes in *in vitro* cultures produce migration inhibitory factor into the supernatant The evidence of spontaneous lymphokine synthesis is strengthened further by this study, which demonstrates leukocyte chemotactic activity in these culture supernatants The factor has a molecular weight of more than 5000 daltons, it resisted heating for 15 min at 100° C, and showed maximum activity at dilution 1 4–1.8 of the supernatants

Key words Leukocyte chemotactic factor, lymphokine

H Arvilommi, Public Health Laboratory, SF-40620 Jyvaskyla 62, Finland

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Lymphocytes, when activated by antigen, can elaborate soluble agents which possess various biological activities The array of these substances, collectively called lymphokines (*Dumonde et al.* 1969), is expanding as more and more factors are being reported on This is true also for agents capable of stimulating lymphocytes into lymphokine synthesis Apart from antigens, various other agents, such as mitogens or allogeneous lymphocytes, may activate cells into lymphokine synthesis (for references see *Oppenheim & Rosenstreich* 1976). We have reported earlier (*Arvilommi & Räsänen* 1975) that unstimulated human lymphocyte culture supernatants contain some principle which inhibits the migration of human leukocytes The data suggested that this activity might be due to leukocyte inhibitory factor (LIF) produced by lymphocytes without any intended stimulus. We extended this line of investigation by the study of other lymphokines possibly synthesized similarly. This paper describes chemotactic activity in the supernatants of human lymphocyte cultures

MATERIALS AND METHODS

Heparinized blood from healthy blood donors or laboratory staff was centrifuged on Ficoll-Isopaque to obtain mononuclear cells (*Böyum* 1968) These cells contained less than 1% granulocytes and 7–22% monocytes Cell cultures were prepared as described previously (*Arvilommi & Räsänen* 1975) and incubated at 37° C or 4° C for three days in 16 × 100 mm tissue culture tubes (Sterilin Ltd, Middlesex, England) at a concentration of 4×10^6 cells/2 ml. The cultures were terminated by centrifugation at 1000 g for 10 min and the supernatants were collected for chemotaxis assay

Leukocyte chemotaxis was assayed by a modification of Boydens'technique (*Boyden* 1962) described by *Arvilommi & Laatikainen* (1977). In brief, double chambers were formed from two disposable leukocyte migration plates (Sterilin Ltd) put against each other The chambers were separated by 8 μ celluloce acetate filters, diameter 20 mm (Millipore Corp, Bedford, Mass., USA.) The upper chambers were filled through holes bored to the upper plate Each chamber takes about 0 45 ml solution In this way, a set of six reversible double chambers was formed from a pair of plates. For the assay, the lower chamber was filled with the solution to be assayed and the upper chamber with leukocyte

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suspension (1 × 10⁶/ml) Leukocytes were prepared from heparinized blood obtained from healthy blood donors and laboratory staff. Nine volumes of blood were sedimented with one volume of 5% dextran T 150 (Pharmacia Fine Chemicals, Uppsala, Sweden) in Hanks' Balanced Salt Solution (HBSS) at 37° C for 30–45 min. Serum and buffy coat leukocytes were collected and the leukocytes were washed three times with HBSS Finally, the cells were suspended in medium 199 (Gibco Bio-Cult Ltd, Renfrewshire, Scotland) containing 10% horse serum (Flow Laboratories, Irvine, Scotland) and antibiotics. The chemotaxis chambers were incubated for 1 h at 37° C, were reverted to avoid detachment of the cells migrated through the filter, and incubated for a further 2 h. Following incubation, the chambers were disassembled and the filters were fixed in 96% ethanol overnight. The filters were stained with Papanicolaou's stain (*Papanicolaou* 1954), cleared with xylene and mounted on microscope slides under a cover glass. All determinations were made in duplicate. The cells migrated through the filter were counted under the microscope by 500 × magnification. The counting area in each field was a rectangle formed by an engraving in the eye piece Two times five fields from each filter were counted and thus four five-field figures were obtained for each assay. The results were expressed as mean ± SD from these four figures. 1% casein in culture medium was used as positive control and culture medium alone served as negative control In some experiments we used L-fucose and N-acetyl-D-glucosamine (Sigma, St. Louis, Mo., U.S.A.) as inhibitors of LIF or migration inhibitory factor (MIF) (*Rocklin* 1976). Calibrated PD-10 columns containing Sephadex G-25M (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were used to separate some culture supernatants into two fractions (over and below 5000 daltons). In order to study the thermostability of the chemotactic activity, the culture supernatants were incubated for 30 min in 56° C or 80° C water baths or 15 min in a 100° C water bath

RESULTS

When mononuclear cells were cultured for three days in medium containing 10% serum at 37° C and 4° C and the supernatants tested for chemotactic activity, the activity in 4° supernatants was similar to that of the medium control. In contrast, 37° C supernatant contained clear chemotactic activity, although the activity was not as great as with casein (Table I). We then tried to dilute off the activity in the supernatants and performed a series of experiments where doubling dilutions of 37° and 4° supernatants were assayed. In 10 experiments the results were consitent, and these are shown in Fig 1. which represents a typical experiment. The 37° C supernatants proved more chemotactic when diluted to 1:4–1:8, the activity exceeding that of positive casein control, while further dilution lowered the activity to the starting level (1:32). This bell-shaped curve was in sharp contrast to the flat curve obtained with 4° C supernatant.

The shape of the curve could be explained on the assumption of the presence of both the LIF and the chemotactic factor. The former would inhibit the leukocytes from responding to the latter, except in higher dilutions where only the latter activity remains detectable. To work out this possibility, we

TABLE I *Chemotactic Activity in Undiluted Culture Supernatants*

Exp no	Supernatants 37° C	Supernatants 4° C	Medium control	1% casein
1	22 ± 8[a]	3 ± 2	5 ± 2	25 ± 8
2	17 ± 3	14 ± 1	16 ± 2	47 ± 3
3	8 ± 6	2 ± 1	5 ± 1	37 ± 5
4	15 ± 4	6 ± 2	3 ± 1	21 ± 6
Mean	15.5	6.2	7 2	32.5

[a] Cells per 5 fields ± SD

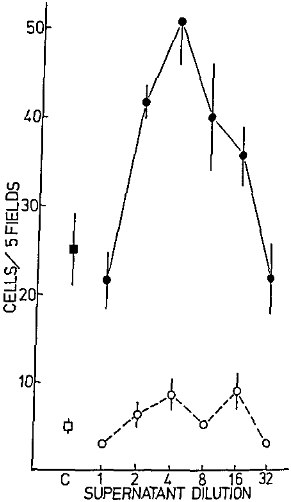


Fig 1 Leukocyte chemotactic activity in dilutions of culture supernatants ● 37° C, and O 4° C supernatants, □ medium control, ■ casein.

tried to block the effect of possible LIF. This was accomplished by heating the supernatants at 100° C for 15 min, a treatment which destroys LIF but preserves chemotactic activity, as shown later. In addition, LIF or MIF was inhibited by N-acetyl-D-glucosamine and L-fucose. However, in all these experiments the results were variable in a way which could not be explained by the hypothesis presented above.

To ascertain whether the activity produced in lymphocyte cultures was really chemotactic and not chemokinetic, i.e. induces directional and not random migration of leukocytes, experiments were designed in which, in addition to the usual set-ups, chambers were prepared where 1:8 dilution of the active supernatant was put in both compartments and thus no chemotactic gradient was formed. As shown in Table 2, the stimulating principle seems to be chemoattractive because, in the absence of gradient, the number of cells migrating through the filter was comparable to that in the controls.

Clearly one possibility as a source of chemotactic activity in culture supernatants might be the presence of serum during culture. It is conceivable that, for example, complement could be activated or otherwise altered, resulting in products which are chemotactic. Therefore it was important to investigate whether chemotactic activity was present in supernatants of cells cultured without serum supplement. When these supernatants were assayed, 10% serum was added after culture, because leukocytes in chemotaxis assay do not migrate in the absence of serum protein. In Fig. 2 the results of a representative experiment show that serum is not needed for elaboration of the chemoattractant.

In an attempt to characterize this lymphocyte derived chemotactic activity, we performed studies to ascertain the thermolability and the approximate molecular weight. These results are given in Table 3. The chemoattractant appeared to be heat stable. It eluted rapidly from a calibrated Sephadex G-25 column. It thus seems to have a quite high molecular weight, at least over 5000 daltons.

TABLE 2 *Differentiation of Chemotactic from Chemokinetic Response*

Exp no	Gradient	No gradient	Control
1	23 ± 3[a]	3 ± 1	6 ± 1
2	67 ± 23	4 ± 2	1 ± 1
3	70 ± 21	7 ± 3	2 ± 1
Mean	53 3	4 6	3 0

[a]Cells per 5 fields ± SD

TABLE 3 *Heat Stability and Molecular Weight of Chemotactic Activity*

Heat treatment	4° supernatant Exp 1	4° supernatant Exp 2	37° supernatant Exp1	37° supernatant Exp 2
Control	3 ± 2[a]	9 ± 4	23 ± 9	79 ± 12
56° C 30 min	7 ± 7		26 ± 14	
80° C 30 min	2 ± 1		16 ± 7	
100° C 15 min	3 ± 1	15 ± 12	31 ± 14	65 ± 31
Gel filtration				
Original supernatant		5 ± 2		62 ± 23
Fraction over 5000 dalton		5 ± 1		70 ± 13
» under » »		2 ± 1		10 ± 4

[a]Cells per 5 fields + SD

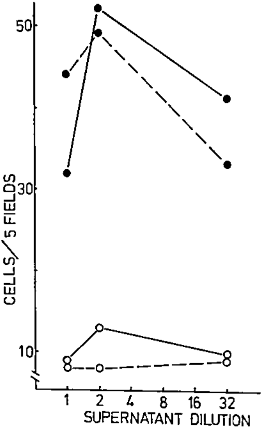


Fig 2 The effect of serum on elaboration of leukocyte chemotactic activity in culture ● 37° C, and ○ 4° C supernatants Solid line. serum free medium, dotted line: medium supplemented with serum.

DISCUSSION

We have shown here that human lymphocytes are able to elaborate leukocyte chemotactic activity during culture, in spite of the fact that no known activating agents were present. When added to our earlier observation on migration inhibitory activity in supernatants of unstimulated cultures (*Arvilommi & Räsänen* 1975), these data support the hypothesis of spontaneous lymphokine synthesis by lymphocytes. We prefer to call this phenomenon spontaneous until the stimulating principle has been identified. However the search for such activator may be unrewarding.

Several studies have shown that when activated with antigen, sensitized lymphocytes are able to synthesize lymphokines which are chemotactic to a variety of white cells (for references see *Remold & David* 1974). The crucial point is whether the leukotactic activity found in this study can be accounted for by a particular chemotactic factor, i.e. a lymphokine. For this activity to appear, metabolic processes in the lymphocytes are needed, since its elaboration is blocked by cold. It is of high molecular weight and its biological activity is undistinguishable from that of a chemotactic lymphokine.

On the other hand, we have ascertained that the leukotactic principle in the cultures cannot be due to serum or cell-to-serum interactions, because no serum is needed for its formation. In conclusion, the data available suggest that chemotactic lymphokine(s) is, in fact, produced by unstimulated human lymphocytes.

One interesting result in the present study was the bell-shaped curve obtained in the dilution experiments. If it is caused by an inhibitor, we were unable to demonstrate that the inhibitor in question is LIF It would make sense that *in vivo* chemotactic factors, when active in high dilutions, are able to attract cells from a long distance, whereas LIF could stop the cells only in the vicinity of antigen-cell interaction since its effect is quickly diluted off. It is also possible that the bell-shaped curve illustrates the rather narrow optimal concentration range of the chemotactic factor. Thus chemotaxis would be inhibited if too high concentrations of the factor were present. At this stage, however, these possibilities remain open.

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DISTRIBUTION OF FOUR SUBCLASS-SPECIFIC LYMPHOCYTE MARKERS IN MAN

ANNAMARI RANKI and PEKKA HÄYRY

Transplantation Laboratory, Fourth Department of Surgery, University of Helsinki, SF 00290 Helsinki 29, Finland

Ranki, A. & Hayry, P. Distribution of four subclass specific lymphocyte markers in man. Acta path. microbiol. scand. Sect. C, 86: 63–71, 1978.

A report is given of the relative and absolute distribution of lymphocytes carrying three T or B cell specific markers in cord blood, in children and in the blood of healthy adult human subjects. The determinations were performed from stained cytological cell smears under conditions where no subclass specific lymphocyte selection or depletion takes place. In adult blood 78 ± 8% (S. D.) ($1.2 \pm 0.4 \times 10^9/l$) of lymphocytes expressed the acid α-naphthyl acetate esterase (ANAE) marker. The percentage distribution of the ANAE marker-carrying lymphocytes was stable in different age groups. The absolute values in cord blood and in young children were approximately three times higher than in adults. A somewhat smaller fraction of adult lymphocytes (53 ± 14%; $1.0 \pm 0.4 \times 10^9/l$) formed rosettes with 2-amino-ethylisothiouronium bromide-hydrobromide (AET) sensitized sheep erythrocytes (AET-SRBC). Although the relative distribution of AET-SRBC rosette-forming lymphocytes was slightly lower in children than in adults, the absolute numbers were approximately two to three times higher. The number of surface Ig-carrying lymphocytes, as detected by rosette formation with *Staphylococcus aureus* strain Cowan I (StaCw) bacteria in adolescent and adult blood was 19 ± 11% ($0.4 \pm 0.2 \times 10^9/l$). The relative number of surface Ig-carrying lymphocytes varied very little in the different age groups. The absolute numbers in cord blood and in small children were approximately twice as high as in adults. The distribution of lymphocytes carrying the fourth marker, surface receptor for IgG, was investigated by antibody-coated human erythrocytes (EA). In adults, the number of lymphocytes forming rosettes with EA was 30 ± 10% ($0.7 \pm 0.3 \times 10^9/l$). Although the relative values were somewhat lower in children, the absolute values were approximately twice as high as in adults.

Key words: Lymphocyte subclasses, esterase.

A. Ranki, Transplantation Laboratory, University of Helsinki, Haartmaninkatu 3, SF 00290 Helsinki 29, Finland

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The quantification of lymphocyte subclasses from blood is gaining important application in clinical medicine. Currently available methods are based on the use of subclass specific surface markers, live cells and phase contrast or fluorescence microscopy (*Jondal et al.* 1973, *Frøland & Natvig* 1973). We have proposed (*Ranki et al.* 1976) an alternative technique by which it is possible to quantify lymphocyte subclasses directly from cytological cell smears. Four markers have been adapted for this procedure up to now. T lymphocytes are detected by demonstration of acid α-naphthyl acetate esterase (ANAE) activity (*Mueller et al.* 1975; *Ranki et al.* 1976) and /or by rosetting them with sheep red cells (*Pellegrino et al.* 1975.), B lymphocytes are detected by rosette formation with *Staphylociccus aureus* strain Cowan I bacteria (*Ghetie et al.* 1974, *Ranki et al.* 1976) and Fc receptor-carrying lymphocytes are detected by the conventional antibody-coated erythrocyte rosettes (*Jondal et al.* 1973). This methodology is devoid of any selective depletion of lymphocyte subclasses (*Häyry et al.* 1977) and, therefore, makes it also possible to quantify absolute numbers of marker-carrying cells in e.g. human blood. As a practical guide for laboratory analysis, a report is given of the relative and absolute distribution of lymphocytes carrying

DISCUSSION

We have shown here that human lymphocytes are able to elaborate leukocyte chemotactic activity during culture, in spite of the fact that no known activating agents were present. When added to our earlier observation on migration inhibitory activity in supernatants of unstimulated cultures (*Arvilommi & Räsänen* 1975), these data support the hypothesis of spontaneous lymphokine synthesis by lymphocytes. We prefer to call this phenomenon spontaneous until the stimulating principle has been identified. However the search for such activator may be unrewarding.

Several studies have shown that when activated with antigen, sensitized lymphocytes are able to synthesize lymphokines which are chemotactic to a variety of white cells (for references see *Remold & David* 1974). The crucial point is whether the leukotactic activity found in this study can be accounted for by a particular chemotactic factor, i e. a lymphokine. For this activity to appear, metabolic processes in the lymphocytes are needed, since its elaboration is blocked by cold. It is of high molecular weight and its biological activity is undistinguishable from that of a chemotactic lymphokine.

On the other hand, we have ascertained that the leukotactic principle in the cultures cannot be due to serum or cell-to-serum interactions, because no serum is needed for its formation. In conclusion, the data available suggest that chemotactic lymphokine(s) is, in fact, produced by unstimulated human lymphocytes.

One interesting result in the present study was the bell-shaped curve obtained in the dilution experiments. If it is caused by an inhibitor, we were unable to demonstrate that the inhibitor in question is LIF. It would make sense that *in vivo* chemotactic factors, when active in high dilutions, are able to attract cells from a long distance, whereas LIF could stop the cells only in the vicinity of antigen-cell interaction since its effect is quickly diluted off. It also possible that the bell-shaped curve illustrat the rather narrow optimal concentration range the chemotactic factor. Thus chemotaxis would inhibited if too high concentrations of the fact were present. At this stage, however, these possib ities remain open.

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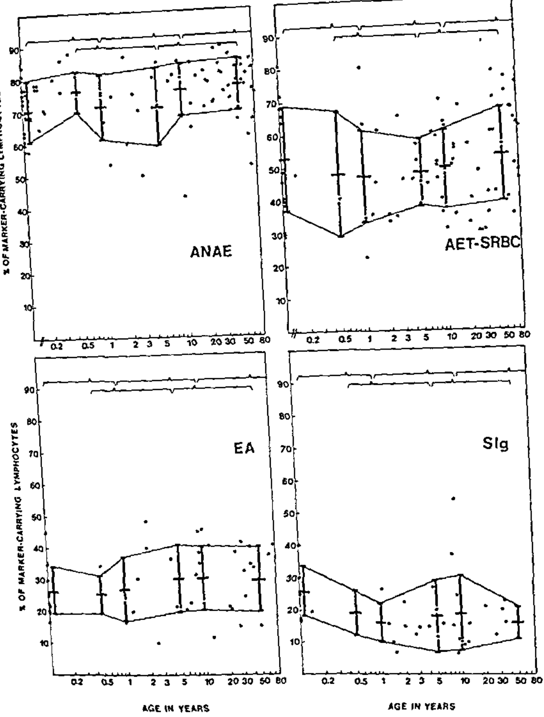


Fig 1 Relative distribution of ANAE marker-carrying lymphocytes, blood lymphocytes forming rosettes with AET-treated SRBC, blood lymphocytes forming rosettes with antibody-coated human O-erythrocytes (EA), and lymphocytes expressing large amoungs of surface Ig and forming rosettes after anti-Ig treatment with *Staphylococcus aureus* Strain Cowan I bacteria. Bars indicate SD. Lines in the upper part of the figure indicate which values were included in each determination of the mean

these four markers in cord blood and in the blood of healthy human subjects from newborn to adults.

MATERIALS AND METHODS

Patients

Patients reporting for elective surgery at Department IV of Surgery or at the Department of Paediatrics, University of Helsinki Hospital, served as blood donors. The diagnoses of the paediatric patients consisted of phimosis, testicular retention, pes equinovarus, congenital luxation of hip and minor anomalies of the fingers and anus. The major diagnoses in ghe adult group were varicose veins, hernias and biliary calculi without evidence of infectious diseas. In addition, samples were occasionally obtained from healthy blood donors of the Finnish Red Cross Blood Transfusion Service. Blood was drawn from all adult donors before surgery. This was also the case with most of the paediatric patients, although some samples were taken five days after the operation or later.

Preparation of Lymphocytes

Venous blood (2–5 ml) was obtained by venipuncture into a heparinized syringe (1:100 Heparin-Medica, Helsinki, no preservative added). Immediately afterwards blood films were made for differential counts and for ANAE staining, and the white cell count was performed with acetic acid. The blood was diluted 1:3 with PBS, layered on Ficoll-Isopaque, density 1.083 g/ml (*Bøyum* 1968), and centrifuged for 30 min at 300 G. The lymphocyte-enriched population was recovered at the interphase and contained approximately 70–80% lymphocytes, 15–25% monocytes and few neutrophilic leukocytes We have found previously that no subclass specific lymphocyte depletion takes place during purification (*Hayry et al.* 1977)

Rosette Procedures

The *sheep erythrocyte rosette-forming lymphocytes* (SRBC-RFL) were quantified by using the 2-aminoethyl-isothiouronium-bromide- hydrobromide (AET) method of *Pellegrino et al* (1975), as reported in detail elsewhere (*Ranki et al.* 1976) One batch of AET-treated indicator cells was good for five days. The rosettes were prepared by incubating 50–100 µl 1% sheep-RBC suspension with 50–100 µl lymphocyte suspension containing 2–4 × 10^6 cells/ml in RPMI-1640 medium The cells were immediately centrifuged in roundbottomed Cooke microtitre plates for 1 min at 200 G After centrifugation, rosettes were suspended gently and the cells were cytocentrifuged onto microscope slides (300 RPM for 5 min) The slides were stained with May-Grunwald-Giemsa (MGG) Lymphocytes binding five or more erythrocytes were counted as rosette-forming lymphocytes

The *Fc receptor-carrying lymphocytes* (EA-RFL) were detected by antibody-coated human-O red cell indicator cells Human red cells were washed with PBS, incubated for 30 min at +37° C in the presence of the first subagglutinating dilution (1.600) of rabbit anti-human red cell IgG, washed three times in PBS and finally diluted into 1% suspension to Dulbecco's BSS. Fifty to 100 µl red cell suspension and 50–100 µl lymphocyte suspension containing 2–4 × 10^6 lymphocytes/ml were distributed onto the microtitre trays and incubated for 15 min at room temperature. After centrifugation (1 min at 200 G) the cells were incubated for a further 15 min at +4° C, the rosettes were disrupted and the cells were centrifuged onto microscope slides. The slides were stained with MGG. Lymphocytes binding four or more erythrocytes were regarded as rosette-forming

Surface Ig-carrying lymphocytes (StaCw-RFL) were identified by rosetting anti Ig-coated lymphocytes with *Staphylococcus aureus* Strain Cowan I (StaCw) bacteria (*Ghetie et al.* 1974; *Ranki et al.* 1976). One hundred µl lymphocyte suspension (4 × 10^6 cells/ml) in Hepes-buffered RPMI-1640 medium containing 0.5% BSA and 0.02% sodium azide were distributed in round bottomed microtitre trays, incubated with 100 µl 1:25 diluted polyvalent rabbit anti-human Ig (anti-IgM, IgG, IgA) for 30 min at +4° C, and washed three times. Then 50 × 10^5 formalin-treated, heat-inactivated StaCw bacteria (a gift from *H. Wigzell*, University of Uppsala) were added with 100 µl per well. The tray was centrifuged for 5 min at 250 G and incubated for 30 min at +4° C. The rosettes were suspended gently and cytocentrifuged onto microscope slides The slides were stained with May-Grunwald-Giemsa. Lymphocytes binding approximately 20 or more bacteria on their surface were registered as positive

Demonstration of ANAE Activity

The demonstration of esterase activity was performed in conditions described by *Müller et al.* (1975) with minor modifications (*Ranki et al.* 1976) Blood films or cytocentrifuged cell smears were incubated in a medium consisting of 40 ml 0.067 M phosphate buffer pH 5 0, 2.4 ml hexazotized pararosaniline, and 10 mg α-naphthyl acetate (Sigma, St. Louis, Mo.) in 0.4 ml acetone. The mixture was adjusted to pH 5.8 using 1 N NaOH. Smears were incubated at room temperature for 8–17 h, washed in distilled water for 10 min, dried and counterstained in 1% aqueous toluidine blue solution for 60 min This counterstaining gave an excellent morphology to the preparations, and lymphocytes were easily distinguished from monocytes or from other white blood cells The quantification of ANAE marker- carrying lymphocytes was performed from cytocentrifuged preparations of Ficoll-Isopaque -purified white blood cells or directly from blood smears. As reported previously (*Hayry et al.* 1977), the results did not differ significantly from each other. A lymphocyte was recorded as ANAE positive if its cytoplasm contained a single or a few distinct spots of the reddish brown reaction product

RESULTS

1. Relative Distribution of the Marker-carrying Lymphocytes in Different Age Groups

The relative distribution of lymphocytes binding AET sensitized sheep red cells (AET-SRBC) on

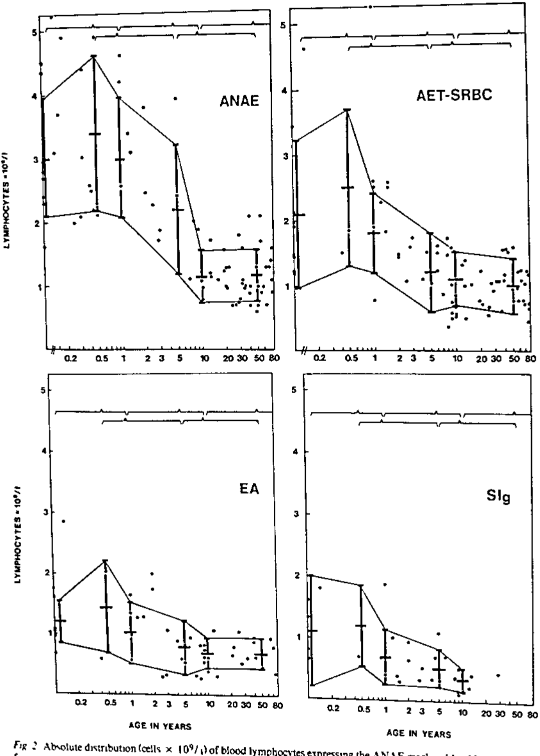


Fig. 2. Absolute distribution (cells × $10^9/l$) of blood lymphocytes expressing the ANAE marker, blood lymphocytes forming rosettes with AET-treated SRBC, blood lymphocytes forming rosettes with antibody coated human O-erythrocytes (EA), and blood lymphocytes forming rosettes after anti-Ig coating with *Staphylococcus aureus* strain Cowan I bacteria. Bars indicate mean ± SD

their surface (Fig. 1) was quite similar in different age groups. The values obtained with adult lymphocytes (53 ± 14 %) were slightly higher than those of children (47 ± 14 %). The difference was not significant (Table 1).

The distribution of ANAE marker-carrying lymphocytes was equally similar and no consistent variation was observed between different age groups (Fig. 1). The fraction of adult lymphocytes expressing the ANAE marker (77 ± 8 %) was significantly (Table 1) larger than the fraction of lymphocytes distinguished by the AET-SRBC marker (53 ± 14 %, $p<0.05$). The individual variation (s.d.) was smaller with the ANAE marker than by using the AET-SRBC method.

Lymphocytes expressing surface immunoglobulin were coated with anti-human Ig and detected by rosetting them with the *Staphylococcus aureus* (StaCw) bacteria. Without anti-Ig treatment, 1–4 % of blood lymphocytes formed »spontaneous« rosettes with StaCw particles. In adolescent and adult blood (Fig. 1), a somewhat smaller fraction of lymphocytes formed StaCw rosettes (19 ± 12 %) than in cord blood (26 ± 8 %). The difference was not significant (Table 1). Percentage variation between the rest of the age groups was small.

The number of lymphocytes expressing the Fc receptor for IgG was quantified by using antibody-coated human erythrocytes (EA) as indicator particles (Fig. 1) The relative distribution of Fc rosette-forming lymphocytes was slightly higher in adolescent and adult blood (30 ± 10 %) than in small children (25 ± 6 %) or in cord blood (27 ± 8 %). The differences were not significant (Table 1).

2. *Absolute Distribution of the Marker-carrying Lymphocytes in Different Age Groups*

We have previously demonstrated that no selective losses of T or B lymphocyte subpopula-

TABLE 1. *Relative Distribution and Statistical Analysis of Lymphocytes Carrying the Various Subclass Specific Markers*

Age (years)	% of marker-carrying lymphocytes[a)] ANAE-L	AET-SRBC-RFL	EA-RFL	StaCw-RFL
cord blood	70 ± 8 (14)	53 ± 17 (12)	27 ± 8 (10)	26 ± 8 (16)
0.5 (0–1)	76 ± 6 (16)	48 ± 19 (6)	25 ± 6 (4)	19 ± 7 (3)
1 0 (0.5–5)	71 ± 10 (13)	47 ± 14 (17)	27 ± 11 (11)	16 ± 6 (13)
5 (1–10)	71 ± 12 (16)	48 ± 11 (27)	31 ± 11 (16)	18 ± 11 (19)
10 (5–50)	76 ± 8 (30)	49 ± 13 (35)	30 ± 10 (21)	19 ± 11 (16)
50 (10–75)	77 ± 8 (36)	53 ± 14 (33)	30 ± 10 (15)	16 ± 5 (8)
	(p<0 05)	(p<0 05)		

a) Mean ± SD, number of determinations given in brackets.
Student's two-directional t-test, p-values higher than 0.05 were considered non-significant, N.S.

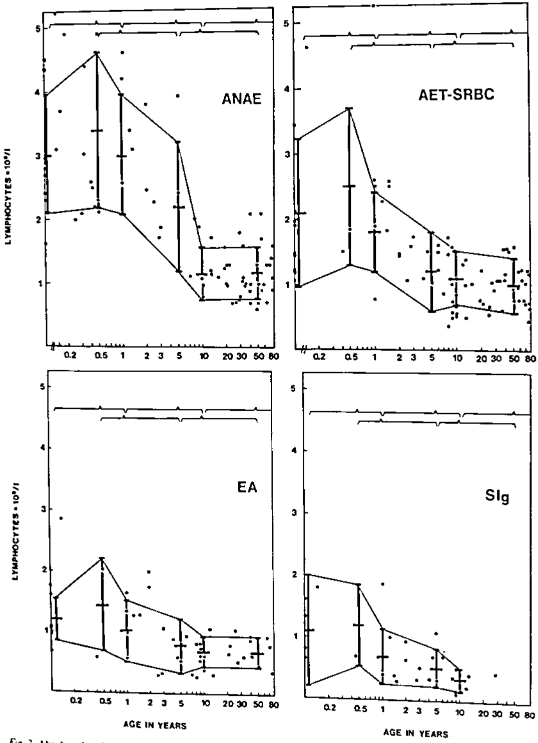


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0 5 (0–1)	76 ± 6 (16)	48 ± 19 (6)	25 ± 6 (4)	19 ± 7 (3)
1.0 (0 5–5)	71 ± 10 (13)	47 ± 14 (17)	27 ± 11 (11)	16 ± 6 (13)
5 (1–10)	71 ± 12 (16)	48 ± 11 (27)	31 ± 11 (16)	18 ± 11 (19)
10 (5–50)	76 ± 8 (30)	49 ± 13 (35)	30 ± 10 (21)	19 ± 11 (16)
50 (10–75)	77 ± 8 (36)	53 ± 14 (33)	30 ± 10 (15)	16 ± 5 (8)

ANAE-L vs AET-SRBC-RFL at 50 (10–75): ($p<0.05$); AET-SRBC-RFL vs StaCw-RFL at 50 (10–75): ($p<0.05$). Comparisons between age groups within each marker: (N.S.)

a) Mean ± SD, number of determinations given in brackets.
Student's two-directional t-test, p-values higher than 0 05 were considered non-significant, N.S.

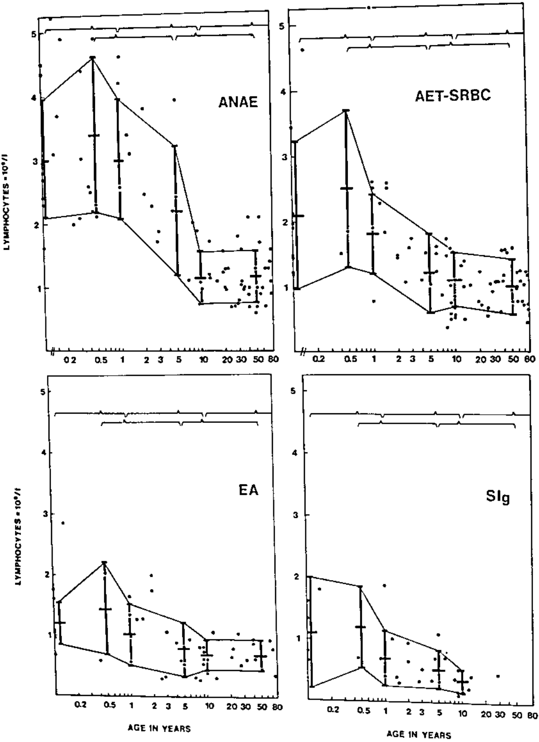


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The number of lymphocytes expressing the F[c] receptor for IgG was quantified by using antibod[y] coated human erythrocytes (EA) as indicat[or] particles (Fig. 1) The relative distribution of F[c] rosette-forming lymphocytes was slightly higher i[n] adolescent and adult blood (30 ± 10 %) than i[n] small children (25 ± 6 %) or in cord blo[od] (27 ± 8 %). The differences were not significa[nt] (Table 1).

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Age (years)	ANAE-L	AET-SRBC-RFL	EA-RFL	StaCw-RFL
	% of marker-carrying lymphocytes[a)]			
cord blood	70 ± 8 (14)	53 ± 17 (12)	27 ± 8 (10)	26 ± 8 (16)
0.5 (0–1)	76 ± 6 (16)	48 ± 19 (6)	25 ± 6 (4)	19 ± 7 (3)
1.0 (0.5–5)	71 ± 10 (13)	47 ± 14 (17)	27 ± 11 (11)	16 ± 6 (13)
5 (1–10)	71 ± 12 (16)	48 ± 11 (27)	31 ± 11 (16)	18 ± 11 (19)
10 (5–50)	76 ± 8 (30)	49 ± 13 (35)	30 ± 10 (21)	19 ± 11 (16)
50 (10–75)	77 ± 8 (36)	53 ± 14 (33)	30 ± 10 (15)	16 ± 5 (8)

Comparisons between age groups within each marker: (N.S.). ANAE-L 50 vs. AET-SRBC-RFL 50: ($p < 0.05$); AET-SRBC-RFL 50 vs. StaCw-RFL 50: ($p < 0.05$).

a) Mean ± SD, number of determinations given in brackets
Student's two-directional t-test, p-values higher than 0.05 were considered non-significant, N.S.

TABLE 3. *Relative and Absolute Distribution of ANAE, AET-SRBC and Surface Ig Marker-carrying Lymphocytes in Male and Female Subjects*

	Age (years)		LYMPHOCYTE MARKER[a)] ANAE-L		AET-SRBC-RFL		StaCw-RFL	
Relative distribution (percentage)	0–5	female	77	± 7 (4)	52	± 7 (4)	16	± 6 (4)
		male	74	± 9 (18) (N S)	46	± 15 (14) (N.S)	16	± 6 (9) (N S)
	5–75	female	76	± 9 (21)	54	± 14 (21)	13	± 2 (4)
		male	78	± 11 (22) (N S)	53	± 11 (22) (N S)	19	± 7 (7) (N S)
Absolute distribution (lymphocytes × $10^9/_l$)	0–5	female	2 9 ± 0 97 (4)		1.7 ± 0 5 (4)		0 5 ± 0 1 (4)	
		male	3 2 ± 1 0 (18) (N.S)		2.2 ± 1.3 (14) (N.S)		0 8 ± 0 5 (9) (N.S)	
	5–75	female	1.3 ± 0 5 (21)		1.0 ± 0 4 (21)		0 4 ± 0 1 (4)	
		male	1 5 ± 0 7 (22) (N S)		1 1 ± 0 4 (22) (N S)		0 5 ± 0.2 (7) (N S)	

a) Mean ± SD, number of determinations given in brackets
Student's two-directional t-test, p-values higher than 0 05 were considered non-significant, N S

DISCUSSION

Many studies have been published reporting a »normal« distribution of the T and B subpopulations in the blood of healthy persons These studies have been based on relatively small materials, usually consisting of one age group only With very few exceptions (*Holm et al* 1975), no control experiments have been included to exclude selective depletion of any one of the subpopulations during the necessary lymphocyte purification procedures. *Holm et al* (1975) and *Häyry et al* (1977) have recently defined conditions where no selection takes place In addition to this, one enzymatical marker probably enabling direct visualization of T lymphocytes from blood films has been discovered (*Müller et al* 1975) and applied to man (*Ranki et al* 1976). In the light of these observations we considered it worthwhile to reinvestigate the absolute and relative distribution of T and B lymphocytes in the blood of healthy human subjects.

The method used in this study enables exact quantification of the number of marker-carrying cells and simultaneous identification of cell morphology. Thus it also excludes errors caused by any contaminating non-lymphocytic cells. The method is especially practical for clinical purposes, since the preparations can be made rapidly and the samples can be stored for later reading.

Most investigators report somewhat higher percentages for SRBC rosette-forming lymphocytes than we do (*Ferguson et al* 1974; *Fleiser et al.* 1975, *Hallberg & Hallberg* 1976; *Smith et al.* 1974; *Campbell et al.* 1974; *Holm et al.* 1975). This is probably due to the fact that our requirements for a »rosette« are somewhat more strict, and that the cytocentrifugation procedure used to process the rosette-forming cells onto microscope slides possibly disrupts some rosettes.

The stability of the percentage distribution of SRBC-RFL in different age groups has also been

tions take place during the purification steps necessary for reading of the results. This makes it possible, by counting the total number of white blood cells and the percentage distribution of lymphocytes, to calculate the absolute values of marker-carrying lymphocytes per unit volume of blood.

Consistent changes in the absolute numbers of marker-carrying lymphocytes were observed in different age groups (Fig. 2). The number of AET-SRBC rosette-forming lymphocytes declined from $2.5 \pm 1.2 \times 10^9/_l$ in small children to $1.0 \pm 0.3 \times 10^9/_l$ in adults, and the number of ANAE marker-carrying lymphocytes from $3.4 \pm 1.2 \times 10^9/_l$ to $1.2 \pm 0.4 \times 10^9/_l$. The changes were highly significant ($p<0.001$, Table 2). A similar decline was also observed with surface Ig-carrying and EA rosette-forming lymphocytes. The number of StaCw rosette-forming lymphocytes declined from $1.2 \pm 0.7 \times 10^9/_l$ in small children to $0.4 \pm 0.2 \times 10^9/_l$ in adolescents and adults, and the number of EA rosette-forming lymphocytes from $1.5 \pm 0.7 \times 10^9/_l$ to $0.7 \pm 0.2 \times 10^9/_l$. Both changes were significant ($p<0.001$, Table 2).

3. Sex Distribution of the Marker-carrying Lymphocytes

Finally, investigation was made as to whether there was any sex difference in the relative or absolute distribution of lymphocytes expressing the ANAE, AET-SRBC or surface-Ig markers. As shown in Table 3, there was no sex difference in the relative numbers, although the absolute numbers of lymphocytes belonging to any one of the lymphocyte subclasses were slightly higher in males than in females. The differences were not significant.

TABLE 2. *Absolute Numbers and Statistical Analysis of Lymphocytes Carrying the Various Surface Markers per Unit Volume of Blood*

Age (years)	MARKER-CARRYING LYMPHOCYTES × 10^9/L[a)] ANAE-L	AET-SRBC-RFL	EA-RFL	StaCw-RFL
cord blood	3.0 ± 0.9 (9)	2.1 ± 1.0 (8)	1.2 ± 0.4 (6)	1.1 ± 0.4 (4)
0.5 (0–1)	3.4 ± 1.2 (17)	2.5 ± 1.2 (8)	1.5 ± 0.7 (7)	1.2 ± 0.7 (4)
1.0 (0.5–5)	3.0 ± 0.9 (13)	1.8 ± 0.6 (16)	1.0 ± 0.5 (15)	0.7 ± 0.4 (13)
5 (1–10)	2.2 ± 1.0 (15)	1.2 ± 0.6 (28)	0.8 ± 0.5 (21)	0.5 ± 0.3 (17)
10 (5–50)	1.2 ± 0.4 (34)	1.1 ± 0.4 (33)	0.7 ± 0.2 (21)	0.4 ± 0.2 (9)
50 (10–75)	1.2 ± 0.4 (39)	1.0 ± 0.3 (36)	0.7 ± 0.3 (14)	N.D.

Statistical comparisons shown in the table: ANAE-L: cord blood vs 0.5 (N.S.); cord blood, 0.5, 1.0, 5 and 10 vs 50 (p<0.001). AET-SRBC-RFL: cord blood vs 0.5 (N.S.); 0.5 vs 50 (p<0.001); 1.0 vs 50 (p<0.001); 5 vs 50 (N.S.). EA-RFL: cord blood vs 0.5 (N.S.); 0.5 vs 10 (p<0.001); 0.5 vs 50 (p<0.001); 1.0 vs 50 (N.S.). StaCw-RFL: cord blood vs 0.5 (N.S.); 0.5 vs 10 (p<0.01); 1.0 vs 10 (N.S.); 5 vs 10 (N.S.). EA-RFL vs StaCw-RFL at 10 (p<0.001); ANAE-L vs AET-SRBC-RFL at 50 (p<0.05); AET-SRBC-RFL vs EA-RFL at 50 (p<0.01); AET-SRBC-RFL vs StaCw-RFL (p<0.001).

a) Mean ± SD; number of determinations given in brackets
Student's two-directional t-test, p-values higher than 0.05 were considered non-significant, N.S.; N.D. = not done

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recorded before (*Ferguson et al.* 1974; *Hallberg & Hallberg* 1976; *Fleisher et al.* 1975), though a rather consistent finding is a slight increase in the percentage of SRBC rosette-forming cells during adolescence and adulthood. This observarion was also made here, although the difference was not significant statistically. *Matsaniotis et al.* (1974) and *Diaz-Jouanen et al.* (1975) have claimed that newborn infants have very low percentages of SRBC rosette-forming cells in their circulation. These observations could not be verified here. When the numbers of ANAE marker-carrying lymphocytes and SRBC rosette-forming lymphocytes were expressed as absolute values per unit volume of blood, the number of lymphocytes carrying either one of these markers was 2.5–3 times higher in cord blood and in newborn than in the adult population. This is consistent with the generally known decrease in the absolute numbers of lymphocytes from newborn to adult life.

Our observations on the number of surface Ig-bearing lymphocytes are compatible with those of several other investigations using immunofluorescence. Most investigators report that approximately 15–18% of SIg-bearing lymphocytes are present in adult peripheral blood (*Holm et al.* 1975; *Frøland & Natvig* 1973; *Campbell et al.* 1974; Ugazio et al. 1974). However, the StaCw rosette method does not have the handicap (easily encountered with the fluorescent method if anti-Ig Fab fragments are not used as FITC-conjugated tracer) that also monocytes binding immune complexes on their surface are included in the counts.

The number of EA rosette-forming lymphocytes was clearly higher than the number of lymphocytes expressing surface Ig. This observation is compatible with the fact that in addition to B lymphocytes (*Basten et al.* 1972) also some T cells (*Basten et al* 1975) possess the Fc receptor, and that a subpopulation of lymphocytes exists which displays the Fc receptor, lacks the ability to bind erythrocytes and does not express Ig on their surface either (*Jondal et al.* 1973).

We consider it important to give both absolute and relative values for T and B lymphocytes in clinical practice, and, when absolute values are given, to report also the corresponding control values for the respective age group.

The authors are grateful to Mrs *Kaija Jansson* for technical assistance The blood samples were kindly provided by Drs *M Kekomäki* and *R Gordin* of the University of Helsinki Central Hospital, and the *Finnish Red Cross Blood Transfusion Service* The study was financed by grants from the *Finnish Medical Research Council*, the *Sigrid Juselius Foundation* and *Lääke Pharmaceuticals*

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THE ROLE OF C1s, C1r AND PROPERDIN IN THE INITIATION OF THE C3b-DEPENDENT FEEDBACK MECHANISM OF THE COMPLEMENT SYSTEM

ULF JOHNSON

The Institute of Medical Microbiology, Department of Immunology, University of Lund, Lund, Sweden

Johnson, U. The role of C1s, C1r and properdin in the initiation of the C3b-dependent feedback mechanism of the complement system. Acta path microbiol scand. Sect. C, *86*: 73–78, 1978

The influence of activated C1s, C1r and properdin in the fluid phase initiation of the C3b-dependent feedback mechanism of the human complement was studied. It was found that $\overline{C1s}$ caused conversion of C3 and factor B in a normal serum, but not in a serum genetically deficient in C4 or in a serum to which Na_2EDTA had been added. When a normal serum was incubated with $\overline{C1r}$ before incubation with $\overline{C1s}$, only C3 was converted, whereas factor B remained in the unaltered native state. Properdin did not influence the $\overline{C1s}$ mediated conversion of C3 and factor B. When activated properdin was added to a properdin-depleted serum, both C3 and factor B were converted. Activated properdin was also incubated with purified C3 and purified C3b. It was shown that C3 was converted to C3b, but C3b was not degraded despite prolonged incubation.

Key words: Complement system, feedback mechanism, C1s, C1r, properdin.

U. Johnson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

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Together with factor B and activated factor D ($\overline{D}$) C3b forms a complex, $C3\overline{bB}$, which enzymatically cleaves C3 into C3a and C3b (16). The complex is self-assembling in the presence of C3b with an increased turnover of C3 as a result. This C3b dependent feedback mechanism serves as an amplification loop in the complement activation and is efficiently controlled by the C3b inactivator, which cleaves C3b into C3c and C3d (19).

Although B and D by definition belong to the alternate pathway of complement activation, there is evidence that also the classical activation pathway can trigger off the C3b dependent feedback (6). However, these investigations were presented without detailed documentation.

It has been postulated that properdin plays a role in the early activation steps of the alternate pathway (7, 17). We have previously reported that activated feedback mechanism. Recently, however, it was claimed that the major role of properdin would be to stabilize generated $C3\overline{bB}$ complexes by preventing decay of factor B (4, 15). This paper describes immunochemical analyses of the interaction between the two complement activation pathways in the fluid phase, especially the effects of activated C1s, C1r and properdin.

properdin can convert purified C3 into C3b thus making possible assembly of the C3b dependent

MATERIALS AND METHODS

Normal human serum (NHS) was obtained from apparently healthy blood donors and was frozen in aliquots at −80° C within 2 hours of collection.

Preparation of Complement Components

Activated C1s was prepared as described by *Haines &*

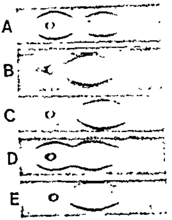


Fig 2 Immunoelectropheresis of factor B Anti-B in the trough and anode to the right A NHS incubated with $C\bar{1}s$ B NHS containing Na_2EDTA, 10 mmoles/l incubated with $C\bar{1}s$ C C4 deficient serum incubated with $C\bar{1}s$ D C4 deficient serum incubated with inulin E NHS

Two µg $C\bar{1}s$ was also added to human serum genetically deficient in C4 Analysis after incubation for 30 minutes at 37° C showed both B and C3 in an unaltered native state (Fig 1 and 2). Inulin treatment of the C4 deficient serum gave a strong conversion of C3 and B indicating a normal function of the alternate pathway These experiments showed that activated C1s cannot react with components in the alternate pathway to generate C3b or to activate B in a C4 deficient serum, even when the alternate pathway is intact, as judged from treatment with inulin Analysis of P in the experiments above by crossed immunoelectrophoresis did not show any detactable shift in the electrophoretic position of the protein compared with an untreated serum

Effect of $C\bar{1}r$

Ten µl of a highly purified preparation of $C\bar{1}r$, containing twice the amount C1r in a pool of normal sera, were incubated with 40 µl of a normal human serum for 30 minutes at 37° C Analysis of C3 and B did not show any conversion of either of the two components (Fig 3 and 4) However, a lowering in the level of macromolecular C1 and a formation of complexes between C1r, C1s and the C1 inhibitor was recorded (13)

Incubation of 40 µl of normal human serum with 10 µl of the C1r preparation at 37° C for 15 minutes followed by incubation with $C\bar{1}s$ at the same temperature for 30 minutes resulted in a

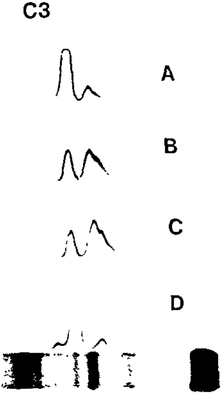


Fig 3 Crossed immunoelectrophoresis with anti-C3 in the gel Anode is to the right A NHS incubated with $C\bar{1}r$ B NHS incubated with $C\bar{1}r$ followed by incubation with $C\bar{1}s$ C NHS incubated with a $C\bar{1}r$-$C\bar{1}s$ complex. D NHS The electrophoretic pattern of normal serum is given for reference

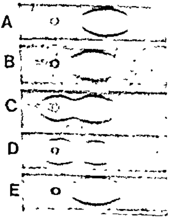


Fig 4. Immunoelectrophoresis of factor B. Anti-B in the trough and anode to the right. A NHS incubated with $C\bar{1}r$. B NHS incubated with a $C\bar{1}r$-$C\bar{1}s$ complex D The same mixture as in B reincubated with inulin. E NHS.

Lepow (8). The preparation had an activity of 397 kU/l as determined by pH-stat titration (11).

Activated C1r was purified according to *Laurell et al.* (12). The preparation was activated by incubation at 37° C for 5 minutes as described by *Ziccardi & Cooper* (23). The preparation contained C1r protein in a concentration twice that of a normal serum pool, as determined by the electroimmuno assay (22).

Activated properdin ($\overline{P}$) was prepared as described previously (9). *C3* was prepared essentially according to *Nilsson & Müller-Eberhard* (18) as described previously (9).

C3b was generated from purified C3 either by mild trypsination or by incubation with $C\overline{42}$ (10).

Activated C1r-C1s complexes were prepared by mixing equimolar amounts of $C\overline{1r}$ and $C\overline{1s}$ in triethanolamine buffered saline (TBS) 0.15 moles/l, pH 7.4, containing Ca^{++} 2 moles/l. The mixture was incubated at 37° C for 15 minutes and the complex formation was tested by crossed immunoelectrophoresis with an antiserum against C1s in the gel as previously described (12). All the C1s was complexed to C1r.

Human serum genetically deficient of C4 was available at the laboratory (*Sjöholm et al.*, to be published).

Antisera

Antisera against C1s, C3 and B were raised in rabbits as described by *Sjöholm* (21). Antiserum against P was obtained as described previously (9).

Affinity Chromatography

Normal human serum was depleted of properdin by affinity chromatography. The IgG fraction of an antiserum against P was coupled to Sepharose 4B by the CNBr method (2). The immunosorbent was packed into a column (1 × 15 cm) and equilibrated with Tris 0.1 mole/l, pH 7.4, containing Na_2EDTA 10 mmoles/l. To 10 ml normal human serum Na_2EDTA was added to the same concentration and the serum was passed over the immunosorbent twice. Before use, the serum was extensively dialyzed against barbital/HCl buffer 75 mmoles/l, pH 7.5, followed by concentration to the original volume with the use of a Diaflo device equipped with a UM-2 membrane. Mg^{++} and Ca^{++} was added to the concentration of 2 mmoles/l, respectively. On immunodiffusion the serum proved immunochemically deficient in P.

Electrophoretic Procedures

C3 conversion products were detected by crossed immunoelectrophoresis (10). The same method was also used for analysis of macromolecular C1 and C1 subcomponents, as described previously (12). Conversion of B was analysed by immunoelectrophoresis according to *Scheidegger* (20).

The electrophoretic mobility of P in agarose was determined by crossed immunoelectrophoresis (5). Both electrophoretic steps were done in a 75 mmoles/l barbital/HCl buffer, pH 8.6, containing Ca^{++} 2 mmoles/l. The second electrophoretic step was performed with a potential of 4 V/cm for 20 hours.

RESULTS

The following experiments were performed to establish whether the C3 convertase assembled by the components of the classical activation pathway, $C\overline{42}$, can initiate the C3b dependent feedback mechanism in fluid phase. Experiments designed to elucidate the role of properdin were also performed.

Effect of $C\overline{1s}$

Two µg $C\overline{1s}$ was added to 20 µl normal human serum and the mixture was kept at 37° C for 30 minutes. Analysis of factor B showed activation to $\overline{B}$, and crossed immunoelectrophoresis of C3 revealed a partial conversion to C3c (Fig. 1 and 2).

When the same amount of $C\overline{1s}$ was added to 20 µl normal serum containing 10 mmoles Na_2EDTA/l, neither B nor C3 were converted (Fig. 1 and 2).

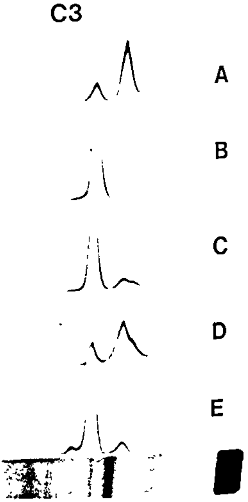


Fig. 1. Crossed immunoelectrophoresis with anti-C3 in the gel. Anode is to the right. A. NHS incubated with $C\overline{1s}$. B. NHS containing Na_2EDTA, 10 mmoles/l, incubated with $C\overline{1s}$. C. C4 deficient serum incubated with $C\overline{1s}$. D. C4 deficient serum incubated with inulin. E. NHS. The electrophoretic pattern of normal serum is given for reference.

DISCUSSION

The present investigation was designed to elucidate the ability of $C\bar{1}s$ and $C\bar{1}r$ to initiate the C3b dependent feedback mechanism in the fluid phase. The participation of properdin in triggering off the feedback was also studied

The results confirm that on incubation with activated C1s, both C3 and factor B were converted to C3c and $\bar{B}$, respectively, which has been reported earlier (6) *May & Frank* in 1973 reported a new complement-mediated cytolytic mechanism which they called the C1-bypass activation pathway (14). They found that heavily sensitized sheep erythrocytes could be lysed in a C4-deficient guineapig serum by activating the complement sequence at the C3 level, thus bypassing C4 and C2 This pathway was probably initiated by the antibody-mediated activation of C1 We were not able to confirm this activation mechanism in the fluid phase. However, it must be stressed that the test systems were not the same When a serum genetically deficient in C4 or a serum to which Na_2EDTA had been added in excess, was incubated with activated C1s, neither C3 nor B was converted. Furthermore, the C4-deficient serum showed B conversion after incubation with inulin which excludes abnormalities of the alternate pathway. Evidently, the C3 convertase, $C\overline{42}$, assembled through activation of the classical pathway, generates C3b which in the presence of factor B and $\bar{D}$ initiates the C3b-dependent feedback mechanism This means that conversion of B to $\bar{B}$ cannot be used to differentiate between classical and alternate pathway activation

When activated C1r was added to normal human serum, a lowering of the level of macromolecular C1 and a complex formation of $C\bar{1}r$, $C\bar{1}s$ and the C1 inhibitor was observed which reflected an activation of macromolecular C1 (12) However, C3 and B were not converted as might be expected from the results where $C\bar{1}s$ was added to serum Further investigations are needed to explain this finding Addition of $C\bar{1}s$ to C1r treated serum did not result in conversion of B or was accompanied by a conversion of a very low grade, while the conversion of C3 was of the same extent as when the serum was incubated with $C\bar{1}s$ alone Incubation of the $C\bar{1}r$ treated serum with inulin resulted in an almost complete conversion of B indicating that the C3 convertase assembled by the alternate pathway was not influenced by C1r. The reason why B is not converted into $\bar{B}$ despite presence of C3b cannot be explained by the present experiments It is known that B might have enzymatic activity without being splitted (1, 3).

It has recently been claimed that the major, and probably the only, role of properdin in the activation of the alternate pathway, is to stabilize the C3 converting enzyme $C\overline{3bB}$ by preventing B to decay (4, 15) In the present investigation, $C\bar{1}s$ is added to a serum in which P has been removed by immune adsorption, and the degree of C3 and B conversion does not differ from what is found in a normal human serum on incubation with $C\bar{1}s$ in the persence of P in physiological amounts. Furthermore, in our hands inulin, which in a normal serum causes a very strong conversion of both C3 and Bupon incubation, did not initiate any conversion of these components when added to a P-depleted serum. We think that these results indicate that P might have another function beside that of stabilizing $C\overline{3bB}$. Evidence for such a function was reported in 1974 by *Götze & Müller-Eberhard* (7) who observed conversion of C3 and B in a normal serum on addition of $\bar{P}$ which was purified to homogeneity. Using highly purified preparations we previously reported that $\bar{P}$ converted C3 to C3b (9). These experiments have been repeated with the use of different preparations of C3 as well as $\bar{P}$. Prolonged incubation of C3b with $\bar{P}$ was not accompanied by any demonstrable fragmentation of the C3b molecule, a fact that strongly suggests that the observed C3 conversion is due to $\bar{P}$ and not to a contaminating protease, since C3b is quite susceptible to proteolytic digestion. Based on these observations we conclude that the activated P does generate C3b from native C3 in a direct manner thus providing C3b for initiation of the C3b-dependent feedback machanism.

This work was supported by grants from the *Swedish Medical Research Council* (Grant B78-16X-00068-14A) and from *Alfred Österlunds Stiftelse*.

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conversion of C3 to C3c. In contrast, B was unaltered or showed a very slight conversion to $\overline{B}$. On reincubation of the mixture with inulin, conversion of B was massive (Fig. 3 and 4).

Effect of $C\overline{1}r$-$C\overline{1}s$ Complexes

Incubation of serum for 30 minutes at 37° C with preformed $C\overline{1}r$-$C\overline{1}s$ complexes corresponding to 2 µg $C\overline{1}s$/20 µl normal serum, showed both C3 and B conversion to the same extent as if only $C\overline{1}s$ was added to the serum (Fig. 3 and 4).

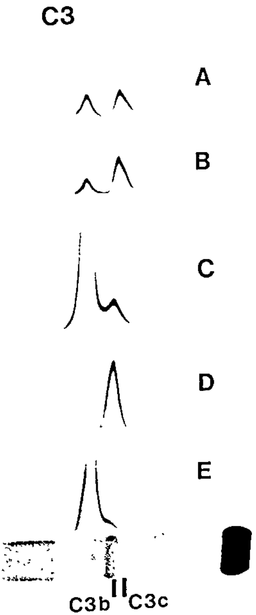


Fig. 5 Crossed immunoelectrophoresis with anti-C3 in the gel. Anode is to the right. A NHS depleted of P incubated with $C\overline{1}s$ B NHS depleted of P incubated with $\overline{P}$. C Purified C3 incubated with $\overline{P}$ D C3b incubated with $\overline{P}$. E. NHS depleted of P incubated with inulin. The electrophoretic pattern of normal serum is given for reference.

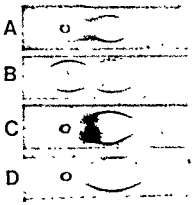


Fig. 6. Immunoelectrophoresis of factor B. Anti-B in the trough and anode to the right. A. NHS depleted of P incubated with $C\overline{1}s$. B NHS depleted of P incubated with $\overline{P}$. C. NHS depleted of P incubated with inulin D NHS

Effect of Properdin

The influence of P in the C1s mediated conversion of C3 and B was investigated with the following experiments. Two µg C1s was added to 20 µl serum depleted of P by immune adsorption, and the mixture was incubated for 30 minutes at 37° C. It was found that C3 and B were converted to approximately the same degree as when C1s was added to an unadsorbed normal serum (Fig. 5 and 6). To exclude the possibility that the observed activity of the $C\overline{1}s$ preparation was due to a contaminating protease, 10 µg of highly purified C3 was incubated with 2 µg $C\overline{1}s$ in the presence of 2 mmoles/1 Mg^{++}. After 30 minutes at 37° C C3 was analysed with crossed immunoelectrophoresis No conversion was noted.

To detect any possible differences in conversion of C3 and B upon incubation of a P depleted serum with $C\overline{1}s$ and inulin, respectively, inulin was added to the P depleted serum used in the $C\overline{1}s$ incubation experiment. Analysis showed that both C3 and B remained in their unaltered native states (Fig. 5 and 6). Addition of highly purified $\overline{P}$ to the P depleted serum resulted in conversion of C3 and B (Fig. 5 and 6).

Twenty µl of the $\overline{P}$ preparation containing about 50 µg $\overline{P}$/ml was added to 20 µl of purified C3 (2 µg/µl), and the mixture was incubated at 37° C for 30 minutes. Crossed immunoelectrophoresis showed a partial conversion of C3 to C3b (Fig. 5) When the same amount $\overline{P}$ (1 µg) was added to 10 µg C3b followed by incubation at 37° C for 30, 60, and 90 minutes, respectively, no degradation of the C3b molecule was detected (Fig. 5).

DISCUSSION

The present investigation was designed to elucidate the ability of $\overline{C1s}$ and $\overline{C1r}$ to initiate the C3b dependent feedback mechanism in the fluid phase. The participation of properdin in triggering off the feedback was also studied.

The results confirm that on incubation with activated C1s, both C3 and factor B were converted to C3c and $\bar{B}$, respectively, which has been reported earlier (6) *May & Frank* in 1973 reported a new complement-mediated cytolytic mechanism which they called the C1-bypass activation pathway (14). They found that heavily sensitized sheep erythrocytes could be lysed in a C4-deficient guineapig serum by activating the complement sequence at the C3 level, thus bypassing C4 and C2. This pathway was probably initiated by the antibody-mediated activation of C1. We were not able to confirm this activation mechanism in the fluid phase. However, it must be stressed that the test systems were not the same When a serum genetically deficient in C4 or a serum to which Na_2EDTA had been added in excess, was incubated with activated C1s, neither C3 nor B was converted Furthermore, the C4-deficient serum showed B conversion after incubation with inulin which excludes abnormalities of the alternate pathway. Evidently, the C3 convertase, $\overline{C42}$, assembled through activation of the classical pathway, generates C3b which in the presence of factor B and $\bar{D}$ initiates the C3b-dependent feedback mechanism This means that conversion of B to $\bar{B}$ cannot be used to differentiate between classical and alternate pathway activation.

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Effect of $C\overline{1}r$-$C\overline{1}s$ Complexes

Incubation of serum for 30 minutes at 37° C with preformed $C\overline{1}r$-$C\overline{1}s$ complexes corresponding to 2 µg $C\overline{1}s$/20 µl normal serum, showed both C3 and B conversion to the same extent as if only $C\overline{1}s$ was added to the serum (Fig. 3 and 4).

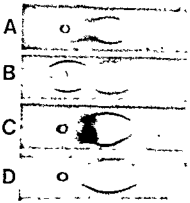


Fig. 6. Immunoelectrophoresis of factor B. Anti-B in the trough and anode to the right. A. NHS depleted of P incubated with $C\overline{1}s$. B. NHS depleted of P incubated with $\overline{P}$. C. NHS depleted of P incubated with inulin D NHS

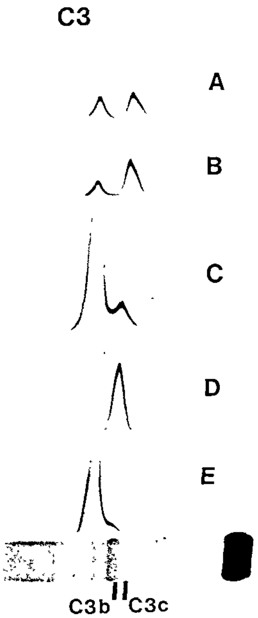


Fig 5 Crossed immunoelectrophoresis with anti-C3 in the gel. *Anode is to the right* A NHS depleted of P incubated with $C\overline{1}s$ B NHS depleted of P incubated with $\overline{P}$. C. Purified C3 incubated with $\overline{P}$ D C3b incubated with $\overline{P}$. E. NHS depleted of P incubated with inulin The *electrophoretic pattern of normal serum is given for* reference

Effect of Properdin

The influence of P in the C1s mediated conversion of C3 and B was investigated with the following experiments. Two µg C1s was added to 20 µl serum depleted of P by immune adsorption, and the mixture was incubated for 30 minutes at 37° C. It was found that C3 and B were converted to approximately the same degree as when C1s was *added to an unadsorbed normal serum* (Fig 5 and 6). To exclude the possibility that the observed activity of the $C\overline{1}s$ preparation was due to a contaminating protease, 10 µg of highly purified C3 was incubated with 2 µg $C\overline{1}s$ in the presence of 2 mmoles/1 Mg^{++}. After 30 minutes at 37° C C3 was analysed with crossed immunoelectrophoresis No conversion was noted.

To detect any possible differences in conversion of C3 and B upon incubation of a P depleted serum with $C\overline{1}s$ and inulin, respectively, inulin was added to the P depleted serum used in the $C\overline{1}s$ incubation experiment. Analysis showed that both C3 and B remained in their unaltered native states (Fig. 5 and 6). Addition of highly purified $\overline{P}$ to the P depleted serum resulted in conversion of C3 and B (Fig 5 and 6).

Twenty µl of the $\overline{P}$ preparation containing about 50 µg $\overline{P}$/ml was added to 20 µl of purified C3 (2 µg/µl), and the mixture was incubated at 37° C for 30 minutes Crossed immunoelectrophoresis showed a partial conversion of C3 to C3b (Fig. 5) When the same amount $\overline{P}$ (1 µg) was added to 10 µg C3b followed by incubation at 37° C for 30, 60, and 90 minutes, respectively, no degradation of the C3b molecule was detected (Fig. 5).

DISCUSSION

The present investigation was designed to elucidate the ability of $C\overline{1s}$ and $C\overline{1r}$ to initiate the C3b dependent feedback mechanism in the fluid phase. The participation of properdin in triggering off the feedback was also studied.

The results confirm that on incubation with activated C1s, both C3 and factor B were converted to C3c and $\overline{B}$, respectively, which has been reported earlier (6) *May & Frank* in 1973 reported a new complement-mediated cytolytic mechanism which they called the C1-bypass activation pathway (14). They found that heavily sensitized sheep erythrocytes could be lysed in a C4-deficient guineapig serum by activating the complement sequence at the C3 level, thus bypassing C4 and C2. This pathway was probably initiated by the antibody-mediated activation of C1 We were not able to confirm this activation mechanism in the fluid phase However, it must be stressed that the test systems were not the same When a serum genetically deficient in C4 or a serum to which Na_2EDTA had been added in excess, was incubated with activated C1s, neither C3 nor B was converted Furthermore, the C4-deficient serum showed B conversion after incubation with inulin which excludes abnormalities of the alternate pathway. Evidently, the C3 convertase, $\overline{C42}$, assembled through activation of the classical pathway, generates C3b which in the presence of factor B and $\overline{D}$ initiates the C3b-dependent feedback mechanism This means that conversion of B to $\overline{B}$ cannot be used to differentiate between classical and alternate pathway activation

When activated C1r was added to normal human serum, a lowering of the level of macromolecular C1 and a complex formation of $C\overline{1r}$, $C\overline{1s}$ and the C1 inhibitor was observed which reflected an activation of macromolecular C1 (12) However, C3 and B were not converted as might be expected from the results where $C\overline{1s}$ was added to serum. Further investigations are needed to explain this finding Addition of $C\overline{1s}$ to C1r treated serum did not result in conversion of B or was accompanied by a conversion of a very low grade, while the conversion of C3 was of the same extent as when the serum was incubated with $C\overline{1s}$ alone Incubation of the $C\overline{1r}$ treated serum with inulin resulted in an almost complete conversion of B indicating that the C3 convertase assembled by the alternate pathway was not influenced by C1r The reason why B is not converted into $\overline{B}$ despite presence of C3b cannot be explained by the present experiments. It is known that B might have enzymatic activity without being splitted (1, 3).

It has recently been claimed that the major, and probably the only, role of properdin in the activation of the alternate pathway, is to stabilize the C3 converting enzyme C3b$\overline{Bb}$ by preventing B to decay (4, 15). In the present investigation, $C\overline{1s}$ is added to a serum in which P has been removed by immune adsorption, and the degree of C3 and B conversion does not differ from what is found in a normal human serum on incubation with $C\overline{1s}$ in the persence of P in physiological amounts. Furthermore, in our hands inulin, which in a normal serum causes a very strong conversion of both C3 and Bupon incubation, did not initiate any conversion of these components when added to a P-depleted serum. We think that these results indicate that P might have another function beside that of stabilizing C3b$\overline{Bb}$. Evidence for such a function was reported in 1974 by *Götze & Müller-Eberhard* (7) who observed conversion of C3 and B in a normal serum on addition of $\overline{P}$ which was purified to homogeneity. Using highly purified preparations we previously reported that $\overline{P}$ converted C3 to C3b (9) These experiments have been repeated with the use of different preparations of C3 as well as $\overline{P}$. Prolonged incubation of C3b with $\overline{P}$ was not accompanied by any demonstrable fragmentation of the C3b molecule, a fact that strongly suggests that the observed C3 conversion is due to $\overline{P}$ and not to a contaminating protease, since C3b is quite susceptible to proteolytic digestion. Based on these observations we conclude that the activated P does generate C3b from native C3 in a direct manner thus providing C3b for initiation of the C3b-dependent feedback machanism

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Acta path microbiol scand Sect C, 86 79–88, 1978.

IMMUNOPROLIFERATIVE ALTERATIONS IN LYMPHOID TISSUES AND LIVER IN MICE STIMULATED WITH *CORYNEBACTERIUM PARVUM*

JØRGEN ELLEGAARD, HENRIK STARKLINT, KNUD KRAGBALLE and NIKOLAY V DIMITROV

University of Aarhus, Department of Medicine and Hematology and Institute of Pathology, Aarhus Amtssygehus, Aarhus, Denmark, and Department of Medicine, Michigan State University, East Lansing, Michigan, USA

Ellegaard, J, Starklint, H, Kragballe, K & Dimitrov, N V Immunoproliferative alterations in lymphoid tissues and liver in mice stimulated with *Corynebacterium parvum* Acta path microbiol scand Sect. C, 86 79–88, 1978

Morphological alterations in the spleen, liver, lymph nodes, thymus, bone marrow and blood were studied in DBA and Balb/C mice treated with *Corynebacterium parvum* The spleen lymph nodes and liver increased in size and weight, whereas thymus showed decrease in weight compared to the control group A marked, early proliferation of monocytes in the bone marrow preceded infiltrations by macrophages in lymph nodes, spleen and liver These infiltrations were closely related to the vascular structures, became nodular and decreased around day 12 after the injection A rapid increase in the number of monocytes and transformed lymphocytes of the peripheral blood were noted almost immediately after the administration of the vaccine No relationship between the degree and duration of the histological changes, strain of mice, and number of inoculations was found It is suggested that the increase in size and weight of the lymphoid organs and the liver is primarily caused by invasion of macrophages, perhaps secondary to a preceding proliferation of monocytes in the bone marrow

Key words *C parvum*, macrophages, granuloma, immunestimulation.

Jørgen Ellegaard, Department of Medicine II (Hematology) Aarhus Amtssygehus, DK-8000 Aarhus C, Denmark

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Corynebacterium parvum (C parvum) and other strains of anaerobic corynebacteriae have been demonstrated to exert a remarkable stimulation upon the reticuloendothelial system resulting in an activated macrophage function and enlargement of liver, spleen and lymph nodes (2, 4, 11, 13, 20). However, the available information about the histopathological alterations following vaccination with *C parvum* is fragmentary The present study was undertaken in order to examine in detail the sequence of histological and cytological alterations in bone marrow, blood, lymph nodes, spleen, thymus and liver in mice using various schedules for administration of the vaccine.

MATERIAL AND METHODS

Male CBA and Balb/C mice, 5–6 weeks old, weighing between 18 and 25 grams were obtained from inbred, pathogen-free stocks at University of Aarhus. The mice were fed with standard mice food and water ad libitum

Injection schedule· *Corynebacterium parvum* vaccine was obtained as a sterile, formalin-killed suspension of the microorganisms in 2 ml ampules (4 mg) from Merieux Laboratories, Lyon, France.

Three protocols with different schedules were used for short term experiments lasting 18 days. In each protocol, twenty-one mice of both strains were injected intraperitoneally with 0 25 ml *C. parvum* vaccine (0 5 mg) In protocol A the mice were injected on day 1 only, in protocol B on days 1 and 3, and in protocol C on days 1, 3 and 5. Twenty-one mice of each strain served as a

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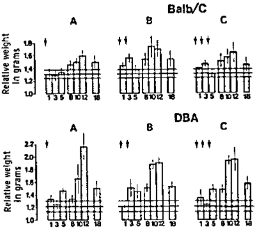


Fig 3 The relative weight of the liver in Balb/C and DBA-mice after injection of *C parvum*. For details, see Fig 1

weight of the livers was noted on day 10 after 2 injections of *C. parvum* (schedule B). In DBA-mice the average increase in the weight of the livers was not related to the number of injections and the maximum increase in liver-weight was noted on day 12 after a single injection. The effect of *C parvum* was still noticeable on day 18 in both strains, but more pronounced in the DBA-mice group In the control mice the relative weight of the livers remained constant

Histological Findings in the Lymphoid Organs and the Liver

Spleen In the injected mice a proliferation of macrophages was found after the third day constituting a mantle zone between the white and the red pulp (Fig 4) This zone was progressing in size reaching a maximum at day 10. At that time all the cells at the margin of the follicles of the white pulp were macrophages The area between this rim of cells and the red pulp contained several types of cells, most of them also macrophages These appeared as mononuclear cells with oval or bean-shaped nuclei and ill-defined cell margins. The nuclear chromatin was finely dispersed without prominent nucleoli The cytoplasm was faint in staining with hematoxylin-eosin, May-Grunwald-Giemsa, and methyl-green pyronin, whereas naphtol AS-D chloroacetate esterase was totally negative (Fig 4 A) The macrophages of the rim at the margin of the follicles and most of the cells in the mantle-zones showed strong positive reaction in frozen sections stained for non-specific esterase (Fig 5 & 5 A), but was negative for fluoride resistant esterase The acid phosphatase reaction was positive in the same areas and cells On the capsule

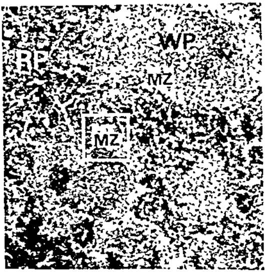


Fig 4 Spleen from DBA mouse stimulated twice with *Corynebacterium parvum* vaccine and sacrified on day 8 Prominent mantle zones are seen between the compartments of the splenic pulp WP = white pulp RP = red pulp MZ = mantle zone (Naphtol AS-D chloro acetate esterase (CAE) (Original magnification × 30)

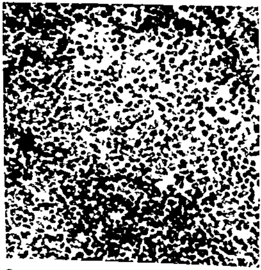


Fig 4 A Detail of mantle zone shown above Most of the cells in the central part of the picture possess the morphological characteristics of macrophages (Original magnification × 200)

aggregates of cells appeared with the same morphological and staining characteristics as described above (Fig 6) From day 12 the border between the mantle zone and the red pulp became indistinguishable. The white pulp itself showed no visible alterations in morphology or cellular composition. Staining of the red pulp with naphtol AS-D

control group receiving no injections, but kept and fed as the injected animals.

According to each protocol, three injected mice and three control mice of each strain were sacrificed on days 1, 3, 5, 8, 10, 12 and 18.

Histological and Cytological Preparation

From all mice bone marrow from the upper end of the femoral bone was immediately smeared and stained by the May-Grunwald-Giemsa technique Blood was drawn from the heart, smeared and stained using the same technique. Microscopic examinations and differential countings of 200 cells were carried out at 1000 × magnification by conventional light microscopy. The intact animals were weighed as were the liver, spleen and thymus after immediate removal. One-third of each spleen was immediately deep-freezed and kept at −70°C. The remaining part of the spleens and the other organs as well as some lynph nodes were fixed in 10% buffered formalin, embedded in paraffin wax and cut in 6 microns thick slices The histopathological features were studied after staining with the haematoxylin-eosin-, the van Gieson-Hansen-, the May-Grunwald-Giemsa and the Gordon-Smith reticulin staining. One animal from each strain, protocol, and day was further studied by staining with methyl-green-pyronin-, and naphtol AS-D chloroacetate esterase technique (14). Reactions of non-specific esterase (17), fluorideresistant esterase (24) and acid phosphatase (14) were investigated on frozen sections of the spleens.

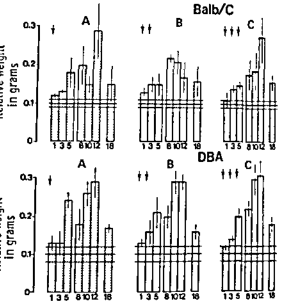


Fig. 1. The relative weight of the spleen, i e. the weight of the organ corrected to a total body weight of 20 grams, in two strains of mice (Balb/C and DBA) after one (A), two (B) and three (C) intraperitoneal injections of *C. parvum* The timing of the injections is indicated by arrows The mean ± SD for the controls is shown by horizontal lines. The number below the columns indicate the days after the first injection of *C. parvum*

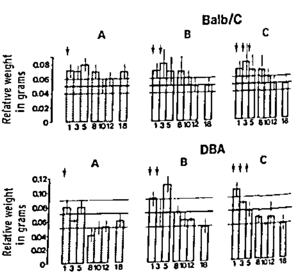


Fig 2. The relative weight of the thymus in Balb/C and DBA-mice after injection of *C parvum* For details, see Fig. 1.

RESULTS

Effect of C. parvum *on the Weight of Organs*

Spleen. The spleens increased remarkably in size and the relative weight, i.e. the weight of the spleen corrected to a total body weight of 20 grams, increased from day 3 and reached a maximum in both strains of mice around day 10–12 after injection of *C. parvum* (Fig. 1). A 200 per cent increase in weight was reached in Balb/C mice after one injection only. No further increase in weight was seen after additional injections in either strain. For the most part the increase in spleen weight was higher in DBA than in Balb/C mice. After 18 days the stimulatory effect of the vaccine had decreased and the weight of the spleens in both strains approximated the control level In the untreated control animals the relative weight of the spleen remained constant throughout the experiment.

Thymus. In contrast to what was found in the spleens, the thymus showed a progressive involution after injection of *C parvum* (Fig 2), this effect being most pronounced after 2 and 3 injections (Schedule B and C) In Balb/C mice a minor increase in the weight of the thymus was initially seen, followed by a rapid decrease, maintained throughout the rest of the experiment. No such pattern was found in DBA-mice where the weight og the thymus decreased from the very beginning, reaching lower levels than the controls at the end of the study. The relative weight of the thymus in the control group remained constant during the first 10–12 days with slight decrease thereafter.

Liver. The livers increased in size and weight after injections of *C. parvum* but the increase was relatively less as compares to that of the spleens (Fig. 3). In Balb/C mice a maximal increase in the

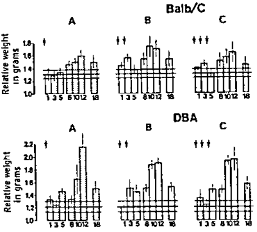


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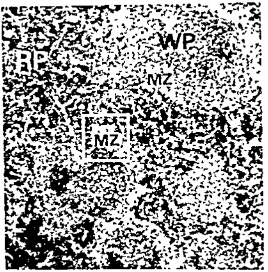


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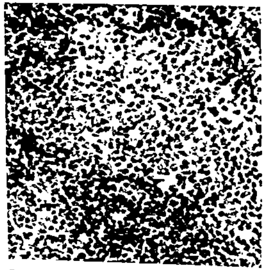


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From all mice bone marrow from the upper end of the femoral bone was immediately smeared and stained by the May-Grunwald-Giemsa technique. Blood was drawn from the heart, smeared and stained using the same technique Microscopic examinations and differential countings of 200 cells were carried out at 1000 × magnification by conventional light microscopy. The intact animals were weighed as were the liver, spleen and thymus after immediate removal. One-third of each spleen was immediately deep-freezed and kept at −70°C. The remaining part of the spleens and the other organs as well as some lynph nodes were fixed in 10% buffered formalin, embedded in paraffin wax and cut in 6 microns thick slices The histopathological features were studied after staining with the haematoxylin-eosin-, the van Gieson-Hansen-, the May-Grunwald-Giemsa and the Gordon-Smith reticulin staining. One animal from each strain, protocol, and day was further studied by staining with methyl-green-pyronin-, and naphtol AS-D chloroacetate esterase technique (14). Reactions of non-specific esterase (17), fluorideresistant esterase (24) and acid phosphatase (14) were investigated on frozen sections of the spleens

RESULTS

Effect of C. parvum *on the Weight of Organs*

Spleen. The spleens increased remarkably in size and the relative weight, i.e. the weight of the spleen corrected to a total body weight of 20 grams, increased from day 3 and reached a maximum in both strains of mice around day 10–12 after injection of *C parvum* (Fig 1). A 200 per cent increase in weight was reached in Balb/C mice after one injection only. No further increase in weight was seen after additional injections in either strain. For the most part the increase in spleen weight was higher in DBA than in Balb/C mice. After 18 days the stimulatory effect of the vaccine had decreased and the weight of the spleens in both strains approximated the control level. In the untreated control animals the relative weight of the spleen remained constant throughout the experiment

Thymus In contrast to what was found in the spleens, the thymus showed a progressive involution after injection of *C parvum* (Fig. 2), this effect being most pronounced after 2 and 3 injections (Schedule B and C). In Balb/C mice a minor increase in the weight of the thymus was initially seen, followed by a rapid decrease, maintained throughout the rest of the experiment. No such

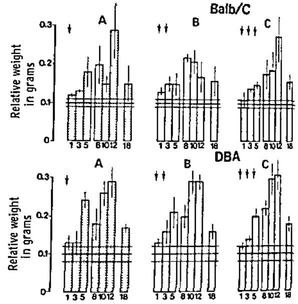


Fig. 1. The relative weight of the spleen, i e the weight of the organ corrected to a total body weight of 20 grams, in two strains of mice (Balb/C and DBA) after one (A), two (B) and three (C) intraperitoneal injections of *C. parvum* The timing of the injections is indicated by arrows The mean ± SD for the controls is shown by horizontal lines. The number below the columns indicate the days after the first injection of *C. parvum*

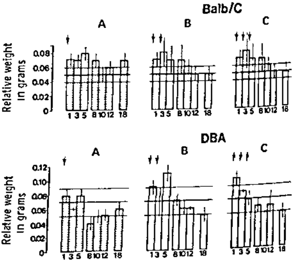


Fig. 2 The relative weight of the thymus in Balb/C and DBA-mice after injection of *C parvum* For details, see Fig 1.

pattern was found in DBA-mice where the weight og the thymus decreased from the very beginning, reaching lower levels than the controls at the end of the study. The relative weight of the thymus in the control group remained constant during the first 10–12 days with slight decrease thereafter.

Liver. The livers increased in size and weight after injections of *C. parvum* but the increase was *relatively less as compares to that of the spleens* (Fig. 3) In Balb/C mice a maximal increase in the

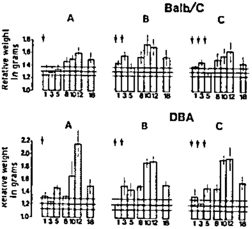


Fig 3. The relative weight of the liver in Balb/C and DBA-mice after injection of *C parvum* For details, see Fig 1.

weight of the livers was noted on day 10 after 2 injections of *C parvum* (schedule B) In DBA-mice the average increase in the weight of the livers was not related to the number of injections and the maximum increase in liver-weight was noted on day 12 after a single injection The effect of *C parvum* was still noticeable on day 18 in both strains, but more pronounced in the DBA-mice group In the control mice the relative weight of the livers remained constant

Histological Findings in the Lymphoid Organs and the Liver

Spleen In the injected mice a proliferation of macrophages was found after the third day constituting a mantle zone between the white and the red pulp (Fig 4) This zone was progressing in size reaching a maximum at day 10 At that time all the cells at the margin of the follicles of the white pulp were macrophages The area between this rim of cells and the red pulp contained several types of cells, most of them also macrophages These appeared as mononuclear cells with oval or bean-shaped nuclei and ill-defined cell margins The nuclear chromatin was finely dispersed without prominent nucleoli The cytoplasm was faint in staining with hematoxylin-eosin, May-Grunwald-Giemsa, and methyl-green pyronin, whereas naphtol AS-D chloroacetate esterase was totally negative (Fig 4 A) The macrophages of the rim at the margin of the follicles and most of the cells in the mantle-zones showed strong positive reaction in frozen sections stained for non-specific esterase (Fig. 4 & 5 A), but was negative for fluoride resistant esterase The acid phosphatase reaction was positive in the same areas and cells On the capsule

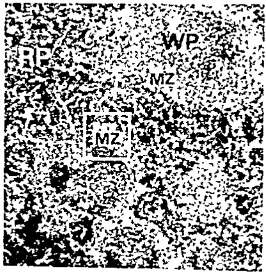


Fig 4 Spleen from DBA mouse stimulated twice with *Corynebacterium parvum* vaccine and sacrified on day 8 Prominent mantle zones are seen between the compartments of the splenic pulp WP = white pulp RP = red pulp MZ = mantle zone (Naphtol AS-D chloro acetate esterase (CAE) (Original magnification × 30)

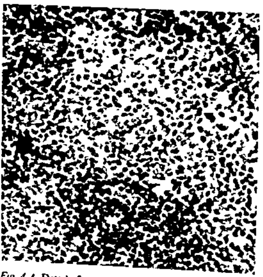


Fig 4 A Detail of mantle zone shown above Most of the cells in the central part of the picture possess the morphological characteristics of macrophages (Original magnification × 200)

aggregates of cells appeared with the same morphological and staining characteristics as described above (Fig. 6) From day 12 the border between the mantle zone and the red pulp became indistinguishable The white pulp itself showed no visible alterations in morphology or cellular composition Staining of the red pulp with naphtol AS-D

control group receiving no injections, but kept and fed as the injected animals.

According to each protocol, three injected mice and three control mice of each strain were sacrificed on days 1, 3, 5, 8, 10, 12 and 18.

Histological and Cytological Preparation

From all mice bone marrow from the upper end of the femoral bone was immediately smeared and stained by the May-Grunwald-Giemsa technique. Blood was drawn from the heart, smeared and stained using the same technique. Microscopic examinations and differential countings of 200 cells were carried out at 1000 × magnification by conventional light microscopy. The intact animals were weighed as were the liver, spleen and thymus after immediate removal. One-third of each spleen was immediately deep-freezed and kept at −70°C. The remaining part of the spleens and the other organs as well as some lynph nodes were fixed in 10% buffered formalin, embedded in paraffin wax and cut in 6 microns thick slices. The histopathological features were studied after staining with the haematoxylin-eosin-, the van Gieson-Hansen-, the May-Grünwald-Giemsa and the Gordon-Smith reticulin staining. One animal from each strain, protocol, and day was further studied by staining with methyl-green-pyronin-, and naphtol AS-D chloroacetate esterase technique (14). Reactions of non-specific esterase (17), fluorideresistant esterase (24) and acid phosphatase (14) were investigated on frozen sections of the spleens.

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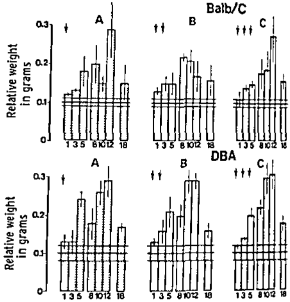


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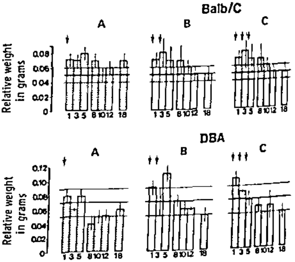


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Thymus. No histological or cytological alterations of thymus were noted after injections with *C. parvum* as compared to non-injected animals. At the end of the experiment, when the mice were about 8 weeks old, no fatty infiltration was visible A quantitative determination of the relative amount of cortical and medullary cells based upon measurements of the width of these zones in serial sections were considered insignificant because of unacceptable high variations in the controls. The number of pyroninophilic cells did not increase after *C. parvum* injections, and in both strains only a few epithelial cells of the medulla and very few inconspicious Hassall-bodies were found. This appearance was unaltered throughout the experimental period with neither formation of epithelial hyperplasia or granulomas, nor cyst formations or other signs of increased secretory activity

Lymph nodes. The lymph nodes became more prominent in all injected mice, but no total dissection and quantitation of the lymph node tissues was attempted Histological alterations were observed in lymph nodes from the thoracic and abdominal cavity from day 3 after the administration of the vaccine. Beginning in the hilar regions nodular formations of macrophages seemed to spread out through the entire lymph node (Fig. 7 & 7 A) These alterations were not related to the number of injections or the strain of mice The cortical and paracortical zones showed no signs of differential lymphatic hyperplasia.

Liver Cellular infiltrations were observed in the liver, beginning from the third day after the first injection with *C parvum* Small groups of mononuclear cells were found in the portal space, closely located to the walls of the interlobar veins (Fig. 8 & 8 A) These mononuclear infiltrations increased gradually in size until day 12 and an extension along the sinusoid wall was observed after day 8 From day 12 a demarcation of the infiltrations with formation of granulomas became evident (Fig. 9 & 9 A). These granulomas were always located close to the vascular walls and were never associated with necrosis or giant cell formation No significant alteration of the number or localization of Kupffer-cells was noted

The number of cells in the infiltrates and in the granulomas showing pyroninophilia increased gradually from day 12 to day 18 after the administration of *C parvum* Single liver cell necrosis was frequently seen, even among the control mice In no case was seen condensation or collapse of the normal pattern of reticulin fibers around the granulomas The number of reticulin fibers within the infiltrates was always very low. The capsule of the liver in the injected mice showed localized

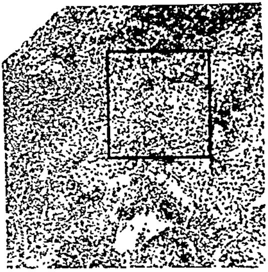


Fig 7 Mediastinal lymph mode from Balb/C mouse stimulated twice and sacrified on day 8. Nodular formations of macrophages in the medulla and paracortical areas (CAE, Original magnification × 80)

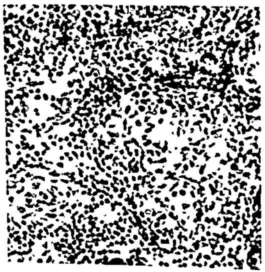


Fig 7 A Detail from above. Macrophages in granulomas (Original magnification × 200)

inflammatory reactions containing the same cellular components as described above. The cellular reactions in the liver after injections with *C. parvum* started earlier and became more pronounced in DBA-mice than in Balb/C mice

Bone marrow and blood Differential countings were performed upon smears of bone marrow and blood from each mouse included in this study (Table 1) In the bone marrow a marked increase in the number of monocytes was noted very early (day 3) after injection with *C. parvum*. Maximum prolifera-

chloracetatesterase revealed a significant increase in the number of granulocytes and their precursors after day 10. At the same time the number of pyroninophilic cells increased in the mantle zones.

No difference between the two strains of mice was demonstrated as to the magnitude of alterations in the spleen, related to the number of *C. parvum* injections.

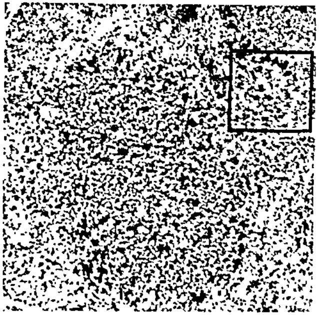


Fig. 5 Spleen from DBA mouse sumulated twice and sacrified on day 8. Frozen section stained for non specific esterase. Positive cells appear as a rim between the white pulp and the mantle. (Original magnification × 80)

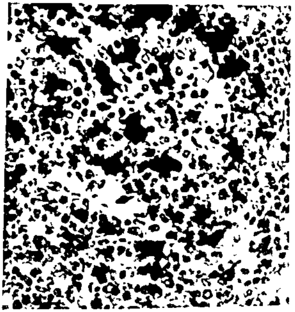


Fig 5 A Detail from Fig. 5. Esterase positive cells dominate in the mantle zone. (Original magnification × 300).

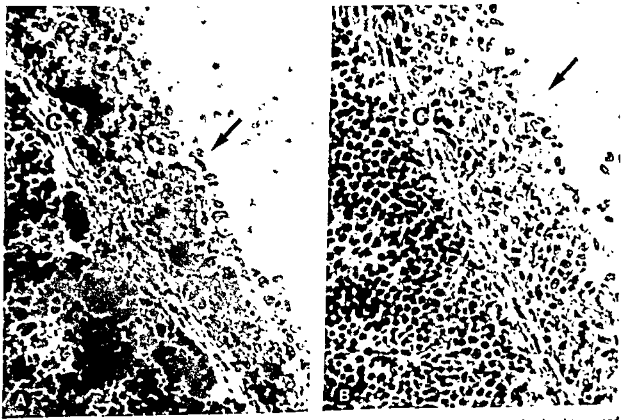


Fig 6 A & B Proliferation of mesothelial cells on the surface of the spleen of Balb/C mouse, stimulated twice and killed on day 8 Most of the subcapsular cells are lymphocytes (A) in which the esterase activity is inhibited by fluoride (B) C = capsule Frozen sections. (Original magnification × 275).

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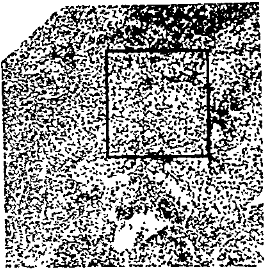


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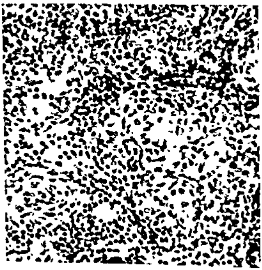


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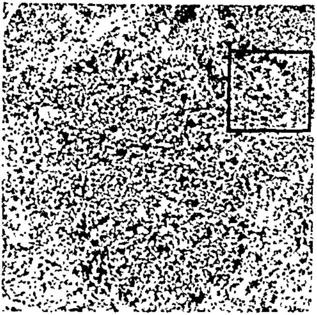


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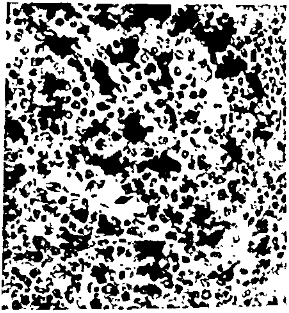


Fig. 5 A. Detail from Fig. 5: Esterase positive cells dominate in the mantle zone. (Original magnification × 300).

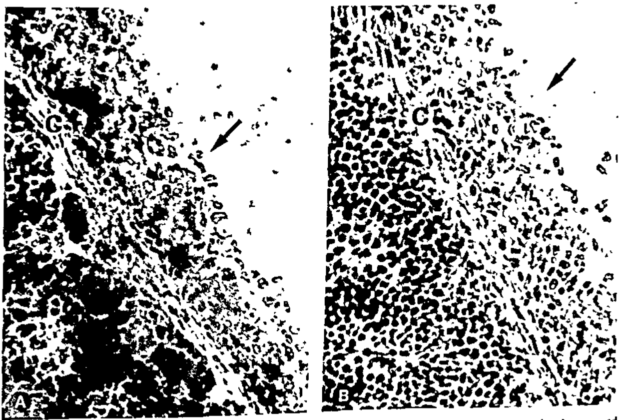


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TABLE 1. *Effect of* C. parvum *on Bone Marrow and Blood*

	Strain	Number of injections	Days after injection of *C. parvum* 1	3	5	8	10	12	18	p-values
Monocytes in the bone marrow	Balb/C	0	0.5	1.5	2.0	2.0	1.0	2.0	1.0	
	–	1	1.0	5.0	4.5	5.0	6.0	5.0	1.0	<0.10
	–	2	2.0	3.0	5.5	6.0	8.0	5.0	1.0	<0.05
	–	3	0.5	5.0	6.0	4.5	10.0	6.0	0.5	<0.05
	DBA	0	0.5	1.0	2.0	1.5	0.5	0.5	0.5	
	–	1	0.5	5.0	5.5	7.0	3.5	5.0	0.5	<0.10
	–	2	0.5	4.0	4.0	8.5	4.0	4.0	0.5	<0.05
	–	3	0.5	4.0	4.0	3.5	3.0	4.5	1.0	<0.05
Monocytes in the blood	Balb/C	0	1.0	2.5	2.0	3.0	3.0	2.0	1.5	
	–	1	2.0	8.0	10.0	12.0	13.0	12.0	3.5	<0.02
	–	2	3.0	7.0	7.0	12.5	14.5	10.0	3.0	<0.02
	–	3	4.0	15.5	6.0	9.5	10.5	13.5	1.5	<0.05
	DBA	0	0.5	2.5	2.5	2.5	2.0	1.0	1.0	
	–	1	1.0	16.0	7.0	5.0	3.5	7.0	2.0	<0.02
	–	2	0.5	8.0	8.0	5.0	3.5	3.5	1.0	<0.10
	–	3	0.5	17.0	4.5	4.0	3.5	3.0	1.0	<0.10
Transformed lymphocytes in the blood	Balb/C	0	3.0	5.0	6.0	5.0	4.5	4.5	5.0	
	–	1	2.5	17.0	16.0	17.0	16.0	33.0	9.5	<0.05
	–	2	3.5	25.0	28.5	30.0	31.0	25.0	26.0	<0.08
	–	3	3.5	24.0	33.0	36.0	38.0	21.0	12.5	<0.02
	DBA	0	4.0	4.0	4.5	4.0	7.5	7.0	4.0	
	–	1	4.0	28.5	18.5	18.5	15.0	12.0	12.0	<0.05
	–	2	4.0	20.0	33.5	32.0	26.0	16.0	15.0	<0.05
	–	3	4.0	14.0	44.0	40.0	36.5	20.0	17.0	<0.05

The percentages of monocytes in the bone marrow and blood, and the relative percentage of transformed lymphocytes, e g the percentage of the total number of lymphocytes in the blood compared to the number of injections and the days after the first injection In each case figures are mean values of differential counting of 200 cells from three mice Calculations for statistical significance by the Wilcoxon test for paired samples

ristic finding in the injected mice was the appearance in the blood of an increased number of plasma cells and cells with all morphological characteristics of macrophages (Fig. 10). These cells were most abundant around day 3 after the administration of the vaccine, and had completely disappeared from the blood at day 18 The number of plasmacells and macrophages in the blood was not dependent on the number of injections, nor on the strain of the mice

None of the mice died from reasons attributable to the injections of the vaccine, and only 2 mice out of the total number of 180 mice were lost during the experimental period

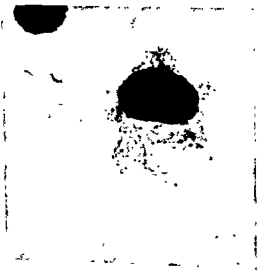


Fig 10 Macrophage in peripheral blood on day 3 (May-Grunwald-Giemsa. (Original magnification × 800)

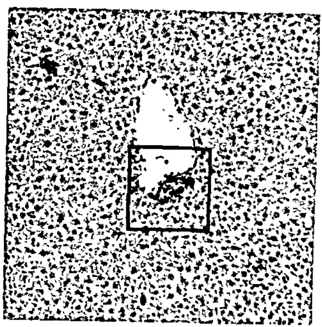


Fig. 8. Liver from DBA mouse stimulated once and killed on day 3. Perivascular and parenchymatous infiltrates (CAE, Original magnification × 80)

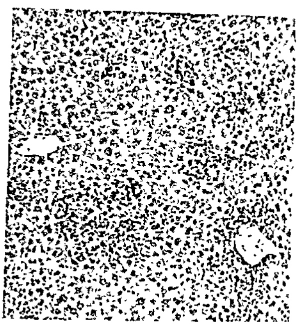


Fig. 9. Liver from Balb/C mouse stimulated twice and sacrified on day 10. The cellular infiltration is increased and demarcated in granulomas. (CAE. Original magnification × 80)

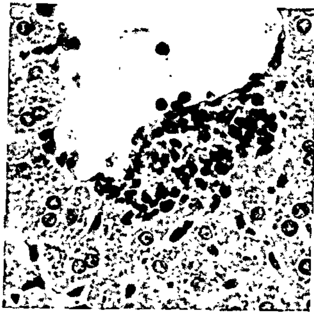


Fig. 8 A. Detail from above. large mononuclear cells constitute the infiltrate. (Original magnification × 300)

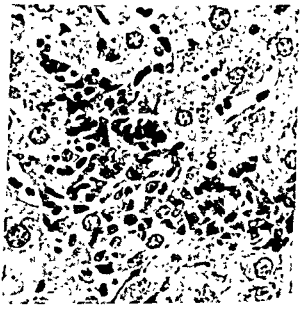


Fig 9 A Macrophages in liver granulomas on day 10, cf. above (Original magnification × 300)

tion of monocytes was usually reached around day 8 after the first injection. The monocytes were morphologically normal with no signs of increased phagocytic or mitotic activity. No changes in the red cell precursors, the granulopoietic- or thrombopoietic cell lines were noted after *C parvum* administration.

In the peripheral blood the relative number of monocytes increased very early reaching a peak around day 3 after the first injection (Table 1) The maximum effect was independent of the number of injections given, but in Balb/C mice the relative number of monocytes in the blood was maintained for a prolonged period after multiple injections with *C. parvum*. The monocytes in the peripheral blood became also more vacuolized and the nucleoli more prominent after multiple injections. An increased percentage of atypical or »transformed« lymphocytes was noted very early in the peripheral blood after injection with *C. parvum*. This effect was more pronounced after multiple injections and reached a maximum between day 5 and 8. Another characte-

the nodular infiltrations observed at the end of the experimental period can be due to a dedifferentiation of the cells into a precursoc stage of macrophages (1)

Castro (4) found that the weight of the axillary and brachial lymph nodes increased in mice which were repeatedly injected with *C parvum* i v. We did not quantitate the total amount of lymph node tissue, but samples of lymph nodes were removed for histological examination. Infiltrations by macrophages similar to those in the spleen and the liver were also found in lymph nodes from various locations No difference between the two strains of mice or the frequency of injections and the magnitude of the alterations in the lymph node tissue could be demonstrated.

The weight of the thymus in Balb/C mice was initially slightly greater after injection of *C parvum* than in the controls Later the thymus in both strains of mice decreased in weight at a rate faster than the controls comparable to the observation by *Greenberg & Dimitrov* (9) under similar experimental conditions Fatty infiltration was not seen during the experiment and no specific histological or cytological alterations were found in thymus in the injected mice The reduction in weight was not likely due to stress as all the animals showed a normal increase in the total body weight, and as the size of the other lymphoid organs increased rather than decreased after injections with *C parvum*

In comparison it has previously been demonstrated that intraperitoneal injection in guinea pigs of various bacterial antigens does not induce change in total body weight or the weight of the thymus (6) Also *Rappaport & Khalil* (18) were unable to demonstrate increase in the thymic weight or gross anatomical alterations in thymus after BCG given intravenously However, they found that BCG injections were followed by pronounced pyroninophilia, increased polyribosomes and increased mitotic activity in the thymic cortical lymphocytes These signs of thymic stimulation occurred early (day 6) after the BCG was given, but were only shortlasting, as every change disappeared within 14 days These findings were interpreted by the authors as an early stimulation of the T-cell proliferation in thymus and a rapid migration of T-cells to other organs, primarily the spleen

The effect of *C parvum* on the immune system appears to be different than that of other immunestimulants, such as BCG (19) An intense, temporary hyperplasia and hyperactivity of the monocyte phagocytic system is induced by BCG as well as by *C parvum*, but the effect by *C parvum* seems to be obtained without the participation of thymus dependent lymphocytes (8, 23) The present study supports this view as no obvious signs of histological alterations in the T-lymphocyte regions of the lymph nodes, spleen and thymus after *C. parvum* injections could be demonstrated.

Our study have demonstrated that *C. parvum* vaccine has a pronounced effect on the monocyte proliferation in the bone marrow and on distribution in various organs and peripheral blood in mice. Such information may be helpful when *C. parvum* is used as an immunomodulator in therapeutic trials for treatment of experimental and human tumors.

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DISCUSSION

The present study has demonstrated that in two strains of mice intraperitoneal administration of a killed suspension of *Corynebacterium parvum* is followed by a sequence of histological and cytological alterations in bone marrow, peripherae blood, spleen, liver and lymph nodes.

The increase in size and weight of the spleens after *C. parvum* injections are comparable to that reported by others (2, 9, 11, 20). As in these experiments the weight of the spleen did not increase further after repeated injections, and maximum for the stimulatory effect was reached around day 12. I contrast *Castro* (4) found a continued rise in the relative weight of the spleen, but this phenomenon is most likely due to the use of a rather high dose of *C. parvum* and to decreasing body weight of the injected mice in his experiments. We found, that a quantitative determination of the relative amount of cells within the red and the white pulp was not possible. The composition of the periarteriolar T-cell zone was investigated after delineation by the method of *Veerman* (21). No alterations in the B- and T-cell zones in the spleen as those described by *Moatamed et al.* (16) after i. v. injections in mice of B- and T-cell mitogens and adjuvants were noted. In the spleen the most important finding after injection of *C. parvum* was the appearance of a mantle-zone of macrophages surrounding the white pulp. Further characterization of the cells, using histochemical procedures for enzymes, characteristic of the histiocytic cell line (24), was in accordance with the cell morphology seen in paraffin sections. The reaction pattern was consistent with monocytes but not with mature macrophages in which the fluorideresistant esterase is positive. This result may indicate that the cells concerned were precursor macrophages (5), or it may be an expression of the immaturity of these enzyme systems in young amimals (15). Decreasing size of the mantle-zone was later accompanied by increase in the number of cells showing pyroninophilia. Proliferation of pyroninophilic cells in the spleen in mice has been reported after immunostimulation by injections of allogenic cells i v (12) and after injection of BCG i. v. (18), but contrary to our results this pyroninophilia was located to lymphocytes in the white as well as in the red pulp. An explanation for this discrepancy could be that immune-modulation of lymphocytes by *C. parvum* is carried out only through the macrophages in the mantle-zones Using a plaque-forming cell assay *Greenberg & Dimitrov* (9) were able to demonstrate that intraperitoneal injections of *C. parvum* in Balb/C mice stimulated the proliferation of IgG and IgM producing cells in the spleen, showing a maximum response around day 13 after the inoculation.

The increase in the size and weight of the liver, which previously has also been reported by others (2, 4, 11), seems to be due to the increased number of macrophages infiltrating the liver tissue. In our study repeated injections of *C. parvum* was not followed by any further increase in the weight of the liver nor was the stimulatory effect prolonged Contrasting to this, *Castro* (4) found that the relative weight of the liver continued to increase in mice injected repeatedly with *C. parvum*. This discrepancy is most likely due to a decrease in the body weight of the mice in Castro's experiments, whereas the injected mice in our study increased their body weight at a rate similar to the controls Also, in contrast to our investigation, sequential histological examination was not carried out systematically in Castro's experiments. We found no increase in the mitotic rate among the infiltrating macrophages, and we are therefore inclined to believe that the infiltration by macrophages may be due primarily to an invasion from the vascular system. This view is strengthened by the demonstration of an early 20-fold increase in the number of monocytes in the bone marrow and the peripheral blood after administration of the vaccine (Table 1), preceding the infiltration by macrophages of the liver and spleen. Additional support comes from the observation that the earliest cellular infiltrations in the liver always were found in close relation to the vascular structures. A mobilization of macrophages is also in accordance with the demonstration that *C. parvum* produces a strong chemotactic factor specific for macrophages (22). In comparison *Rappaport & Khalil* (18) also found histiocytic granulomas in the liver adjacent to vascular structures in mice injected i. v. with BCG. These granulomas were preceded by appearance of aggregates of lymphocytic cells in the portal tracts and in the sinusoids, and the authors considered these granulomas specific for the immune modulation by BCG given i v. By using intraperitoneal injections we were not able to confirm previous findings (2, 10, 11) that multiple injections of *C. parvum* i. v. causes a hyperplasia of the Kupffer-cell system in the liver This discrepancy might be due to either differences in the dose or the route of administration of the vaccine A stimulatory effect of the particulated matter should be considered as well (3) The lack of reticulin formation within the nodular cell infiltrations is in accordance with the classification of macrophages and their precursor cells (7). A collapse of the normal, delicate reticulin mesh in the surrounding liver tissue was never noted. The increased pyroninophilia of the cells in

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BRIEF REPORT

IN VITRO EFFECTS OF RUBELLA VIRUS, STRAIN RA 27/3, ON HUMAN LYMPHOCYTES

III Inhibition of Mitogen Stimulation Transferred by Supernatants from Virus-Infected Cultures

Rolf Maller and Lars Sörén

Departments of Clinical Bacteriology and Infectious Diseases, Linköping University, S-581 85 Linkoping, Sweden

Maller, R & Soren, L *In vitro* effects of rubella virus, strain RA 27/3, on human lymphocytes III. Inhibition of mitogen stimulation transferred by supernatants from virus-infected cultures Acta path microbiol scand Sect. C, 86 89–91, 1978

When lymphocytes from humans with serological immunity against rubella were incubated with live rubella virus, supernatants were obtained which when added to non-infected lymphocytes gave a significant inhibition of phytohaemagglutinin (PHA)-induced proliferation In contrast, supernatants from virus-infected lymphocytes from donors lacking immunity against rubella gave no inhibition of the PHA response

Key words Rubella virus, lymphocytes *in vitro* supernatants, mitogen stimulation.

Rolf Maller, Regionsjukhuset, Linkoping, S-581 85 Linkoping, Sweden.

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Live rubella virus (L-RV) inhibits the PHA-stimulation of normal lymphocytes *in vitro* (8, 10) We have previously shown that this inhibition is immunologically specific (8) i e the inhibition was obtained only with lymphocytes from donors with serological immunity against rubella However, L-RV does not induce a general anergy of lymphocytes *in vitro*, since lymphocytes from rubella-immune individuals incubated with attenuated L-RV can be stimulated to antigen-specific proliferation (9)

The mechanism of the inhibition is, however, not known In the present study we investigated whether the inhibiting effect could be transferred with supernatants from virus-infected, immune lymphocytes to non-infected, non-immune lymphocytes subsequently stimulated with PHA

Materials and methods

Fifty-five healthy volunteers with rubella haemagglutination-inhibiting antibody (HI) titres (2) from < 5 to 320 were used as lymphocyte donors Attenuated rubella virus (L-RV), strain RA 27/3, 10^7 pfu/ml (8, 12), was suspended in »virus buffer« (VB) – 0 025 M phosphate buffer, pH 7 3 with EDTA 0 003 M. When applicable, L-RV was UV-inactivated (UV-RV) for 10 min (9)

Lymphocytes were separated from newly-drawn venous blood (8) and suspended in TCM 199 with 15 per cent heat-inactivated homologous rubella-antibody free AB serum in a concentration of $1.0 - 1.5 \times 10^6$ cells/ml The cells were dispensed in 1 8 ml portions in round bottom glass tubes (primary cultures), to which were added 0 2 ml aliquots of either L-RV (group A), UV-RV (group B) or VB (group C)

Following incubation for 3–6 days, two lymphocyte cultures from each group were pulsed for 18 hours with 0 15 μCi ^{14}C-thymidine (^{14}C-TdR) and the incorporated radioactivity was measured by scintillation counting (9) The antigen-specific stimulation by L-RV was expressed as lymphocyte stimulation index (LSI_{lrv}), i e. cpm per ml cells incubated with L-RV/cpm per ml cells incubated with VB

The remaining tubes of groups A–C were centrifuged at 2500 rev/min for 10 min and the supernatants were collected and pooled within each group. The group A supernatants were either left untreated or inactivated by UV-irradiation, performed in open petri-dishes at 254

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TABLE 2. *PHA-stimulation of Secondary Lymphocyte Cultures Supplemented with Supernatants from Non-immune Primary Cultures Incubated for Different Periods of Time after Addition of Live Rubella Virus*

Primary cultures			Secondary cultures		
			^{14}C-TdR incorporation when incubated with		
n	Incubation time	$LSI_{(rv}$[a])	Sup A	Sup C	Sup A/Sup C
2	3 days	0.9 ± 0.1	10.2 ± 5.9[b]	9.7 ± 4.1[b]	1.02 ± 0.18
5	4 »	0.8 ± 0.2	10.0 ± 3.5	10.0 ± 3.3	0.99 ± 0.07
3	5 »	1.6 ± 0.1	9.5 ± 2.4	9.9 ± 2.9	0.97 ± 0.07
7	6 »	1.2 ± 0.5	9.7 ± 2.8	10.0 ± 2.5	0.97 ± 0.16
17		1.1 ± 0.4	9.8 ± 3.0	10.0 ± 2.7	0.98 ± 0.11

[a]) *Lymphocyte stimulation index after stimulation with live rubella virus*
[b]) cpm/ml × 10^3 ± SD

Several explanations for the observed inhibitory effect of supernatants from L-RV infected cultures are conceivable. Lymphokines with inhibiting effects on PHA-stimulation may be produced in the primary cultures. Interferon, for example, has been shown to inhibit PHA-stimulation of lymphocytes (7). The production of certain lymphokines during incubation of lymphocytes with viral antigens is immuno-specific, but the amount produced is not correlated to the titre of humoral antibodies (5, 11) or to the extent of the antigen-specific lymphocyte proliferation (3, 11). Lymphokines may be heat stable (1, 13). Another possibility is that complexes between *in vitro* produced antibodies and rubella antigen are formed: such complexes can inhibit PHA-stimulation of lymphocytes (6). Finally, the antigen-specific lymphocyte proliferation induced by L-RV might lead to an exhaustion of the medium in the primary cultures. This possibility cannot be dismissed at once, but it seems unlikely, since there was no correlation between the degree of the inhibiting effect of the supernatants and the degree of lymphocyte proliferation in the primary cultures. Furthermore, no inhibition was obtained by supernatants from primary cultures incubated with UV-RV (supernatant B) in which a similar degree of antigen-specific lymphocyte proliferation was recorded.

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nm for 1 hour (4). The group B and C supernatants were left untreated. In some experiments, group A supernatants were heat-inactivated at 56°C for 1 hour (4).

The supernatants were then added in 0.8 ml aliquots to 1.0 ml suspensions of newly-prepared lymphocytes, 2.0×10^6 cells/ml from donors with HI-titres <5 (secondary cultures).

Finally 0.2 ml leucoagglutinin-phytohaemagglutinin (PHA), 50 µg/ml, was added giving an optimal PHA-concentration of 5.0 µg/ml. The cultures were incubated for 3 days, pulsed with ^{14}C-TdR for 5 hours, and the incorporated activity measured.

The results are expressed both as cpm/ml of the secondary cultures (Table 1 and 2) and as the quotients supernatant A/supernatant C and supernatant B/ supernatant C, which is the incorporated radioactivity in the cultures incubated with the supernatants A and B, respectively, divided by the incorporated radioactivity in the cultures incubated with supernatant C.

For statistical evaluation Student's t-test for paired and unpaired observations was used.

Results and discussion

In 33 experiments where supernatants were collected from L-RV infected primary cultures of lymphocytes (supernatants A) from rubella-immune donors (HI-titres 10–320), the PHA-stimulation of secondary cultures incubated with these supernatants was clearly lower than that of secondary cultures supplemented with supernatants from non-infected primary cultures (supernatant C) The mean of the quotients supernatant A/ supernatant C was 0.81 ± 0.31. However, the possible transfer of live virus from the primary cultures might complicate the interpretation of the results. Therefore, in the further analysis UV-inactivated supernatants from virus-infected cultures were studied. In the same 33 experiments (Table 1), the thymidine incorporation of secondary cultures incubated with UV-inactivated supernatant A was significantly lower than that of secondary cultures supplemented with supernatant C ($p < 0.001$) and the mean of quotients UV-inactivated supernatant A/supernatant C was 0.79 ± 0.19 which is equal to the above value of 0.81 ± 0.31 for untreated supernatant A/supernatant C. This indicates that the suppressive effects of untreated supernatants A were accounted for by factors other than live virus. On the other hand, when the primary cultures were incubated with UV-RV, no difference of thymidine incorporation between secondary cultures incubated with supernatant B and supernatant C was observed (results not presented), and the mean quotient supernatant B/supernatant C was 0 96 ± 0 22

When the primary cultures were made up of lymphocytes from non-immune donors (HI-titres <5), no difference of thymidine incorporation between secondary cultures incubated with UV-inactivated supernatant A and supernatant C was observed (Table 2)

These results show that supernatants from primary cultures infected with L-RV inhibited the PHA-stimulation of secondary cultures, and that the generation of the inhibitory factor (s) was immunologically specific. However, the degree of inhibition was not correlated to the HI-titres of the lymphocyte donors or to the degree of the antigen-specific stimulation obtained in the primary cultures.

The results shown in Table 1 suggest that the generation of the inhibitory factors in the supernatants is time-dependent, since inhibitory supernatants were not obtained regularly from primary cultures incubated for less than 4 days.

In 18 experiments, the effects of heat inactivation and UV-inactivation of supernatants from L-RV infected cultures were compared. Similar results were obtained with supernatants treated either way, and the means of the quotients UV-inactivated supernatant A/supernatant C and heat-inactivated supernatant A/supernatant C were 0.73 ± 0.19 and 0.80 ± 0.21, respectively.

TABLE 1 *PHA-stimulation of Secondary Lymphocyte Cultures Supplemented with Supernatants from Immune Primary Cultures Incubated for Different Periods of Time after Addition of Live Rubella Virus*

	Primary cultures		Secondary cultures			
			^{14}C-TdR incorporation when incubated with			
n	Incubation time	LSI$_{lrv}$[a]	Sup A	Sup. C	Sup. A/Sup. C	p-value[b]
7	3 days	1 7 ± 1 5	11 0 ± 2 2[c]	11.9 ± 1.4[c]	0 93 ± 0 15	p>0 2
5	4 »	3 1 ± 1 7	5 4 ± 5 8	7 0 ± 6 0	0 62 ± 0 25	p<0 05
5	5 »	2 8 ± 1 4	5.2 ± 4 1	6.7 ± 3 8	0.74 ± 0.26	0 1>p>0 05
16	6 »	3 6 ± 3 4	6 2 ± 4.4	7.5 ± 5 0	0 81 ± 0 11	p<0 005
33		3.0 ± 2 7	7 0 ± 4 6	8 3 ± 4 7	0 79 ± 0 19	p<0 001

a) Lymphocyte stimulation index after stimulation with live rubella virus
b) Significance of difference Sup. A – Sup. C
c) cpm/ml $\times 10^3$ ± SD

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MITOGEN STIMULATION AND DISTRIBUTION OF T- AND B-LYMPHOCYTES DURING NATURAL RUBELLA INFECTION

ROLF MALLER, ARIL FRYDÉN and LARS SÖRÉN

Departments of Infectious Diseases and Clinical Bacteriology, Linköping University Hospital, Linköping, Sweden

Maller, R., Frydén, A. & Sörén, L. Mitogen stimulation and distribution of T- and B-lymphocytes during natural rubella infection Acta path microbiol scand Sect. C, *86*: 93–98, 1978.

The phytohaemagglutinin (PHA) response of lymphocytes from seven patients with natural rubella infection was investigated during the acute, early and late convalescence stages of the disease When the lymphocytes were cultured in autologous serum, a moderate depression of the response, following stimulation with PHA in both optimal and suboptimal concentrations was obtained two weeks after the onset of exanthema (early convalescence) Two months later (late convalescence), the lymphocyte stimulation response had returned to almost normal values. On the other hand, lymphocytes incubated in pooled homologous serum reacted normally to PHA in optimal concentrations at all three stages Determination of T- and B-lymphocytes did not reveal any change in the relative proportion of T-lymphocytes during the course of the disease. However, in late convalescence, a significant decrease in the relative number of B-lymphocytes was recorded

Key words Rubella infection, mitogen stimulation, T- and B-lymphocytes.

R Maller, Regionsjukhuset, Linköping, S-581 85 Linköping, Sweden

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T-lymphocyte functions become affected in certain viral infections (28) Thus, rubella virus is known to inhibit the blastogenic response of lymphocytes induced by phytohaemagglutinin (PHA) Such lymphocyte hyporeactivity has been demonstrated in the congenital rubella syndrome (19), in natural rubella (2), after rubella vaccination with attenuated virus (7, 16, 25), and after experimental infection with virulent rubella strains in animals (3) and humans (16). Addition of live rubella virus to normal lymphocytes *in vitro* inhibits the PHA response of these cells (15, 18, 21). The mechanism of the virus-induced lymphocyte anergy is not known The degree of inhibition in *in vitro* experiments seems to be correlated with the immune status of the lymphocyte donor against rubella Thus, lymphocytes from donors with high antirubella antibody titres were strongly inhibited, whereas lymphocytes from non-immune donors were not inhibited at all (15). This suggests that the inhibition may be mediated by an immunologically specific mechanism.

Earlier investigations have yielded variable results concerning the timing and degree of inhibition of the PHA response following viral infection. In the vaccination studies, the variable results could be due partly to differences in the virulence of the attenuated strains used (16, 21). It is therefore of interest to investigate the depression of PHA-reactivity induced *in vivo* by virulent rubella strains. To our knowledge there are few reports (and these include only sporadic cases) concerning mitogen stimulation of lymphocytes during the course of natural rubella infection (2, 26). This paper deals with the PHA-stimulation response of lymphocytes from seven patients with rubella during the acute and convalescence stages of the disease. In order to analyse possible roles of humoral factors, the lymphocytes of the rubella patients were cultured in both autologous serum and in pooled homologous serum from persons without serological immunity

serum The same batch of HS was used for both rubella and control lymphocytes The cell concentration was 1 × 10^6/ml Leucoagglutinin-phytohaemagglutinin (PHA) from Pharmacia AB, Uppsala, Sweden, diluted to different concentrations (100, 50, 25 and 10 μg/ml) in physiological saline was added in 0.1 ml aliquots to 0 9 ml lymphocyte suspensions Four aliquots of 0 2 ml from each cell suspension were dispensed on Micro Test II tissue culture plates 3040 (Falcon Plastics, Oxnard, USA), which were closed and incubated at 37°C in humidified air for four days Five hours before the end of the incubation period, 0 03 μCi of ^{14}C-TdR in 0 02 ml saline was added to each culture Cultures were washed and precipitated onto glass fibre filters by a semi-automatic multiple sample processor (24) The filters were placed in 10 ml Permablend III (Packard Instrument Co, Ill, USA) The incorporated radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter and expressed as cpm/ml of cell suspension

Identification of lymphocyte subpopulations Immunoglobulin-bearing lymphocytes were determined (6, 27) using a commercially available polyvalent goat antihuman immunoglobulin fluoresceinisothiocyanate conjugate from Behringwerke Marburg-Lahn, Germany In order to avoid non-specific attachment of aggregated IgG to Fc-receptors on cells other than immunoglobulin-bearing lymphocytes, the antihuman immunoglobulin was subjected to ultracentrifugation before use (5)

Two million lymphocytes were incubated with 0 2 ml immunoglobulin conjugate diluted 1 4 in PBS The vial was kept dark and cool for 30 minutes and was then shaken gently The cells were washed three times and resuspended in PBS The cells with surface fluorescence were considered to be immunoglobulin-bearing *B-lymphocytes* At least 200 small lymphocytes were counted by combined UV- and phase contrast microscope

T-lymphocytes were determined by the E-rosette method (11) Sheep red blood cells (SRBC), stored in Alsever's solution, were washed in PBS adjusted to a 1% suspension in foetal calf serum (FCS) and layered on top of 2 5 × 10^6 lymphocytes The cells were spun down incubated for 15 minutes at 37°C and then kept for 2 hours at +4°C in an ice-bath Resuspension was performed on a rocker (8) One drop of cell suspension was transferred to a slide and covered with a coverslip Lymphocytes binding three or more SRBCs were considered to be T-lymphocytes At least 200 small lymphocytes were counted in duplicate preparations and the mean value was calculated All preparations were read by the same technician

Statistical methods Student's t-test for paired and unpaired observations was used

RESULTS

PHA Stimulation

Table 2 shows the thymidine incorporation at optimal PHA concentration of lymphocytes from seven rubella patients following PHA stimulation in *autologous* serum With the exception of one patient (E K), the PHA response of lymphocytes from rubella patients during the acute stage was quite similar to that of lymphocytes from the control group However, during early convalescence the thymidine incorporation of rubella lymphocytes was significantly lower than that obtained during the acute stage of the disease ($p < 0\ 05$) and that of control lymphocytes ($p < 0\ 05$) At late convalescence, a slight depression of the PHA response was still observed When the lymphocytes were cultured in pooled *homologous* serum (Table 3), no depression of the PHA stimulation was observed at any of the three stages of the disease, the thymidine incorporation of the lymphocytes of the patient group being quite similar to that of the lymphocytes of the control group Fig 1 shows the dose-response

TABLE 2. *Thymidine Incorporation in Autologous Serum of PHA-stimulated Lymphocytes from Rubella Patients during Acute Disease and Convalescence*

Patient	Acute stage	Early convalescence	Late convalescence
S A.	25 7[a]	16.9[a]	15.3[a]
E.K.	7 2	10 8	13 1
Å N	20 3	8 6	19.2
M N	20 4	11.7	19.8
M L.	19 0	18.0	21.3
V L	16 3	9 6	14 0
C S	25 4	20 6	15.8
Mean ± SD	19 2 ± 6 3[b]	13 7 ± 4 7[b]	16 9 ± 3 2[b]

[a] cpm/ml × 10^3
[b] cf control group 19 4 ± 5 0 (n = 8)

TABLE 3 *Thymidine Incorporation in Homologous Serum of PHA-stimulated Lymphocytes from the Same Rubella Patients as in Table 2*

Patient	Acute stage	Early convalescence	Late convalescence
S A.	39 8[a]	25.4[a]	18.3[a]
E.K	10 8	14 7	21.5
Å N	22 1	22 0	19.2
M N	16.8	15 8	17.8
M L.	23 9	24 2	20 0
V L.	17 7	11 6	18.2
C S	24 8	25.3	20 6
Mean ± SD	22.3 ± 9.1[b]	19 9 ± 5 7[b]	19.4 ± 1.4[b]

[a] cpm/ml × 10^3
[b] cf control group 19.3 ± 7.7 (n = 8)

against rubella . Furthermore, the relative proportion of B- and T-lymphocytes during the course of the disease was studied.

MATERIALS AND METHODS

Rubella patients: The material consists of nine patients (two not included in the PHA-study) aged 15–36 years (average 22 years) with clinical signs and humoral antibody titres consistent with rubella (Table 1). They were studied on three occasions: a) during the acute stage, i.e. within five days after the onset of exanthema; b) during the early convalescent stage, i.e. two weeks after the onset of exanthema, when the immune response against rubella is readily detected; and c) during the late convalescent stage, i.e. two months later.

Control groups. Healthy volunteers, two women and six men, aged 22–52 years (average 32 years), were used as lymphocyte donors for the control cultures. The control group in the lymphocyte subpopulation studies consisted of 39 blood donors.

Testing and treatment of sera. Haemagglutination inhibition antibodies and complement fixation antibodies against rubella were determined, using standard methods (4, 23). Rubella antibody-free, blood group AB sera were collected from blood donors, pooled and frozen. Autologous sera (AS) and pooled homologous serum (HS) were inactivated at 56°C for 30 minutes before use.

Lymphocyte separation. Newly drawn venous blood, either heparinized (T- and B-cell studies) or defibrinated by shaking with glass beads (PHA-stimulation studies), was diluted 1:3 in phosphate buffered saline (PBS) and centrifugation was made on Ficoll-Isopaque (1). The separated lymphocytes were washed three times in PBS and suspended in medium (see below). The final cell suspensions contained more than 90% mononuclear cells and the viability checked by trypan blue exclusion was more than 90%.

Lymphocyte cultures. Lymphocytes from rubella patients and controls suspended in TCM 199F (GIBCO, Grand Island, New York), supplemented by 15% of either autologous (AS) or pooled homologous (HS)

TABLE 1. *Data of Patients with Clinical Diagnosis of Rubella*

Patient	Sex & age	Serological test	Titres at different stages of rubella: Acute	Early convalescence	Late convalescence	
S.A.	M 21	HI	< 5 (2)[a]	160	160	Family contacts
		KB	•	•	•	
E.K.	M 19	HI	160 (5)	160	160	
		KB	•	•	10	
Å.N.	F 17	HI	20 (3)	320	320	
		KB	•	•	•	
M.N.	F 24	HI	40 (3)	80	80	
		KB	< 5	10	20	
M.L.	F 36	HI	40 (5)	40	40	Family contacts
		KB	< 5	10	10	
V.L.	F 15	HI	160 (4)	160	160	
		KB		10	10	
C.S.	F 15	HI	20 (4)	160	80	
		KB	•	10	10	
M.A.[b]	F 24	HI	160 (5)	160	160	
		KB	< 5	10	10	
C.B.[b]	M 28	HI	20 (4)	160	80	
		KB	•	•	•	

[a] Day after the onset of exanthema
[b] PHA-stimulation not performed
• = not determined

TABLE 4. *Relative Numbers of B- and T-lymphocytes of Rubella Patients during Acute Disease and Convalescence*

Patient	Acute stage		Early convalescence		Late convalescence	
	B-cells %	T-cells %	B-cells %	T-cells %	B-cells %	T-cells %
S A	4 0	77 0	5.5	72 0	9.0	71.0
E K.	10 5	79.5	9 5	74.0	5 0	66.5
Å N.	14 0	53 5	16.5	67.5	6.0	83 0
M N	4 5	67.0	9 0	69 0	6 0	73 5
M L.	6 5	87.5	5 5	43 0	2 5	79.5
V L	13 0	83 5	7 0	79 0	7 0	84 5
C S	6 5	70.0	7.5	63 5	5.5	74 0
M A	13.5	73 5	7.0	71.5	8.0	74.5
C B	8 0	65 5	6 0	56.5	2.0	62 5
Mean ± SD	8.9 ± 3 9[a]	73 0 ± 10 4[b]	8 2 ± 3 4[a]	66 2 ± 10 8[b]	5 7 ± 2.3[a]	74 3 ± 7.2[b]

[a] cf control group 9.5 ± 4 9 (n = 39)
[b] cf control group 72 0 ± 6 8 (n = 39)

the present study was observed as early as on the third day after exanthema At the end of two weeks the PHA response had returned to almost normal The reason for the discrepancy between these results and our own is not clear but differences in culture conditions might be of importance.

In the present experiments, the depression of the PHA response was most pronounced when the lymphocytes were incubated in autologous serum. Similar observations have been reported concerning the PHA response of lymphocytes from patients with influenza (10) When the lymphocytes were incubated in pooled homologous control serum, the PHA response was depressed only after stimulation with suboptimal amounts of PHA, which agrees with earlier findings on PHA stimulation following vaccination with rubella virus (25)

The mechanism for the rubella virus-induced depression of the PHA response is not clear and its physiological implications are obscure. The present results, showing a decreased PHA reactivity during early convalescence and earlier findings of a PHA hyporeactivity for several weeks after vaccination with rubella virus (7, 16, 25), suggest that the inhibition of PHA response is not connected with the viraemic stage of the disease This means that the inhibition is not the result of viral cytotoxicity or viral interference with the lymphocyte stimulation process However, rubella virus infects lymphocytes during the disease (3, 22) and although they do not die these virus-infected lymphocytes may be unresponsive to PHA stimulation (3, 18) Another explanation may be that the viral infection leads to the production of a substance which inhibits or cooperates with other factors in the inhibition of stimulation This factor might, for example, be an antibody-antigen complex (13) or a lymphokine (14). The production of lymphokines is known to be maximal rather late during viral infections (7, 9, 17, 20) The present finding of a more pronounced depression in autologous serum is in accordance with such an explanation Small amounts of inhibiting substance remaining on the cells incubated in pooled homologous serum would be able to inhibit the stimulation by suboptimal amounts of PHA, which would explain the shift of the PHA dose-response curve for rubella lymphocytes in this serum (Fig 1B)

The percentage of T-lymphocytes did not change significantly during the course of rubella disease, which is in accordance with earlier results (12) However, a significant decrease in the relative proportion of B-lymphocytes at late convalescence was found The implications of this finding are yet to be elucidated

The authors wish to thank Mrs *Ulla Strand*, Miss *Kristina Groth*, Miss *Kristina Orselius* and Mrs. *Gretel Berg* for skilful technical assistance

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curves for PHA stimulation of rubella and control lymphocytes following cultivation in media with autologous and homologous sera. These curves were constructed on the basis of the mean

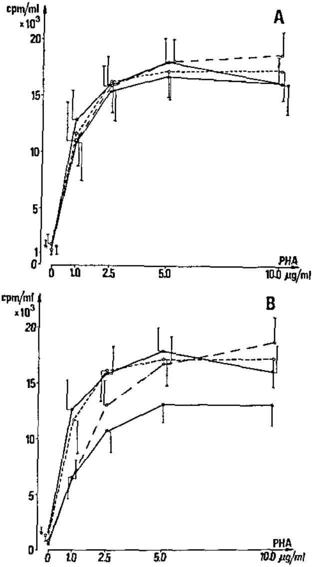


Fig. 1. Dose-response curves for PHA stimulation of rubella and control lymphocytes incubated in autologous serum (AS) or pooled homologous serum (HS) The mean ^{14}C-TdR incorporation of lymphocytes from seven rubella patients and eight controls after stimulation with PHA in indicated concentrations is shown

Fig 1A – acute stage
Fig 1B – early convalescence

● ——— ● lymphocytes from rubella patients in AS

○ —··— ○ lymphocytes from rubella patients in HS

✱ ——— ✱ lymphocytes from controls in AS

✡ - - - - - ✡ lymphocytes from controls in HS

Vertical bars denote standard error of the mean

thymidine incorporation of lymphocytes from the seven rubella patients and the eight controls, respectively, following stimulation with different amounts of PHA. During the acute stage (Fig 1 A), the dose-response curves for rubella and control lymphocytes were identical. In early convalescence (Fig. 1B), however, the thymidine incorporation of rubella lymphocytes incubated in AS was considerably lower than that of control lymphocytes at all PHA concentrations. When lymphocytes from rubella patients during early convalescence were incubated in HS, the thymidine incorporation of these cells was clearly lower than that of control lymphocytes following stimulation with PHA in suboptimal concentrations (Fig. 1B), whereas no such inhibition was seen after stimulation with PHA in optimal concentrations.

Lymphocyte Subpopulations

No significant variation in the absolute numbers of lymphocytes was seen during the course of the disease, and the mean numbers of mononuclear cells were 1.8 ± 0.7; 2.0 ± 1.0; and $2.0 \pm 0.7 \times 10^9/l$ at the acute stage, early and late convalescence stages, respectively. Table 4 shows the relative numbers of B- and T-lymphocytes in the blood of rubella patients at the three stages of the disease. Evidently the relative proportion of T-lymphocytes did not change during the course of the disease; the slight reduction seen in early convalescence was not statistically significant. However, the mean percentage of B-lymphocytes seen at late convalescence, 5.7 ± 2.3, was significantly lower than the mean percentage at the acute stage, 8.9 ± 3.9 ($p < 0.05$), as well as lower than mean percentage of control lymphocytes, 9.5 ± 4.9 ($p < 0.005$).

DISCUSSION

A transient and moderate depression of the PHA response of blood lymphocytes during the course of rubella disease was demonstrated in this study. This is in accordance with the results of other studies on the PHA response following experimental infection with rubella (3, 16) and following vaccination with attenuated rubella virus (7, 16, 25). Other parameters of cell-mediated immunity are also affected during rubella (7, 9, 12, 17). In the present investigation, the depression of the PHA response did not coincide with the acute stage of the disease but was demonstrable after about two weeks, i.e in early convalescence Of course, the maximum depression might have occurred at any time between five days and two weeks. In a recent study on four cases with rubella disease (2), a depression of the PHA response more pronounced than that seen in

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ISOLATION AND PARTIAL CHARACTERIZATION OF A MITOGENIC LECTIN FROM *LATHYRUS ODORATUS* SEEDS

JAN KOLBERG

Department of Immunology, National Institute of Public Health, Oslo, Norway

Kolberg, J Isolation and partial characterization of a mitogenic lectin from *Lathyrus odoratus* seeds Acta path microbiol scand Sect. C, *86*: 99–104, 1978

A mitogenic lectin has been isolated from saline extract of *Lathyrus odoratus* seeds by $(NH_4)_2SO_4$ precipitation and subsequent chromatography on DEAE-Sepharose and CM-Sepharose. The isolated lectin was almost homogeneous by SDS gel electrophoresis and gel filtration, but multiple bands were obtained on isoelectric focusing The molecular weight, determined by gel filtration, was 37,000 Gel electrophoresis in the presence of SDS gave a molecular weight of 19,000, thus suggesting that the lectin is a dimer It agglutinated human erythrocytes of the different ABO groups equally well Human lymphocytes were exposed to the lectin or PHA, and the incorporation of thymidine was measured Compared with PHA, which stimulated maximally in the concentration range of 0 25–1 μg/ml, the *L odoratus* lectin had optimal activity at concentrations of 35 μg/ml and higher.

Key words Lectin, mitogen, lymphocytes *in vitro*

Jan Kolberg, National Institute of Public Health, Postuttak, Oslo 1, Norway

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During the last decade, the number of mitogenic lectins has increased markedly About 15 have been obtained in purified form (13) Apparently most of the lectins exert their agglutinating activity by binding to carbohydrate residues on the cell surface (16) The use of purified agglutinins and mitogens has therefore become a valuable tool for studying polysaccharides, glycoproteins and cell surfaces

The present paper reports mitogenic activity of a purified lectin from seeds of *Lathyrus odoratus* (LATH) belonging to the *Leguminosae* family *Gold & Balding* (5), in reviewing plant and animal lectins, observed that the presence of blood group non-specific lectin in these seeds has been known since 1909

MATERIALS AND METHODS

Extraction and fractionation with $(NH_4)_2SO_4$ The seeds of *Lathyrus odoratus* (LATH) were obtained from Norwegian Seeds A/S 0 25 kg seeds were allowed to swell overnight in 0 9 per cent NaCl at 4° C The material was then suspended in 1 l of 0.9 per cent NaCl and homogenized at maximum speed in an Omnimixer (Sorvall) at 0° C in five sequences of 1 min with intervals to prevent rise in the temperature The homogenate was centrifuged at 13,000 × g for 30 min and the pellet was discarded The proteins contained in the buffy-brown supernate were fractionated by salting out with solid $(NH_4)_2SO_4$ Precipitates obtained by 0–30, 30–60 and 60–100 per cent saturation were spun down and suspended in distilled water and dialyzed against distilled water at 4° C The precipitates formed in the dialysis tubings were separated by centrifugation and discarded The clear supernatants were stored at −20° C.

Chromatography on ion exchangers at 18° C. A mitogenic lectin was separated from the 30–60 per cent $(NH_4)_2SO_4$ fraction by successive chromatography on DEAE-Sepharose CL-6B and CM-Sepharose CL-6B (both from Pharmacia Fine Chemicals AB, Sweden), as described in legends to Figs 1 and 2.

Polyacrylamide gel electrophoresis Continuous sodium dodecyl sulphate (SDS) electrophoresis was performed as described by *Skehel & Schild* (17) Discontinuous slab electrophoresis was performed as described by *Laemmli* (9) Human serum albumin, (mol wt. 68,000), trypsin (mol wt. 23,300) and lysozyme (mol wt. 14,300)

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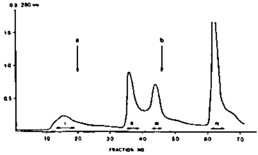


Fig 1 Chromatography on DEAE-Sepharose of the fraction obtained by 30–60 per cent saturation of the seed extract by ammonium sulphate. Protein (200 mg) in 25 ml of 0 1 M of Tris-HCl pH 8 0 was applied to a column (2 5 × 30 cm) equilibrated with the same buffer. This buffer was also used for elution of unbound proteins The arrows indicate stepwise application of buffer of increasing ionic strength- a, Tris-HCL + 0.1 M NaCl, b, Tris-HCl + 0.2 M NaCl Fractions of 9 ml were collected at a flow rate of 45 ml/h

horesis (Fig 3) Both fraction I from DEAE-Sepharose and fraction I B (further purification on CM-Sepharose) showed one band in the continuous system (not depicted) Similar results were obtained in the absence of 2-mercaptoethanol. However, in the discontinuous system, fraction I showed one major band, and four very faint bands, three of which were slow-moving and one fast-moving

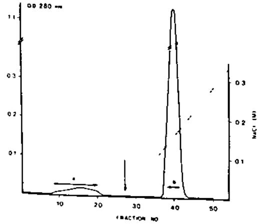


Fig 2 Chromatography on CM-Sepharose of the protein fraction I (Fig 1) Protein (20 mg) in 40 ml 0 1 M acetate buffer pH 4 9 was applied to a column (1 5 × 30 cm) equilibrated with the same buffer This buffer was also used for elution of unbound proteins The arrow indicates the application of a linear NaCl gradient in acetate buffer Fractions of 5 ml were collected at flow rate of 15 ml/h

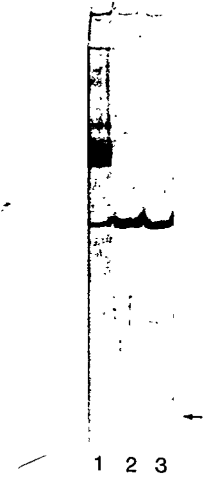


Fig 3 SDS polyacrylamide electrophoresis with 3 per cent stacking gel and 20 per cent separation gel. The arrow marks the position of the tracking dye. 1. Protein fraction corresponding to 30–60 per cent saturation of the seed extract by ammonium sulphate. 2. Fraction I from DEAE-Sepharose 3 Fraction I B from CM-Sepharose

Fraction I B, from the chromatography on CM-Sepharose, revealed the major band and the very faint one with high mobility. The apparent molecular weight of the major band was determined to be 19,000.

Gel filtration Fig 4 shows the elution pattern of the lectin upon filtration on Bio-Gel P-300. The peak of the haemagglutinating activity was coincident with the major peak The mitogenic activity was also found in the material eluted in this peak. The molecular weight of the main component was 37,000. The minor component and the faint band on SDS gel electrophoresis had similar molecular

were used as markers for the molecular weight estimation.

Gel filtration. A column of Bio-Gel P-300 was operated under neutral conditions (0.1 M Tris-HCl pH 8.0). Normal human serum and cytochrome C were used for calibration. Thus the first peak indicated the void volume (V_0), the second peak eluted corresponded to mol.wt. 150,000 (IgG), the third peak corresponded to mol.wt. 68,000 and the fourth contained cytochrome C (mol.wt. 12,400).

Isoelectric focusing. The analysis was performed in a 110 ml LKB column, using an Ampholine gradient pH 3.5–10.

Haemagglutination test. The agglutinating activity was assayed by performing a twofold serial titration of the lectin in saline, starting with a concentration of 1 mg/ml. One drop of lectin solution and one drop of a 2.5 per cent solution of thrice washed human red cells were mixed. After incubation for 2 h at 37° C, the agglutination end points were read microscopically. Fractions from the columns were assayed by an Autoanalyzer technique (8, 10).

Lymphocyte cultures. Human lymphocytes were isolated from heparinized (without preservative) blood, according to the method of *Bøyum* (1) The cells accumulated at the interface were washed thrice with Hanks' balanced salt solution and finely resuspended in Eagle's Minimum Essential Medium supplemented with 10 per cent heat-inactivated human AB serum, penicillin and streptomycin. The cells were cultured in round-bottomed glass tubes (100 × 12 mm) with loosely fitting plastic cups. The tubes, containing cells in 2.0 ml medium, were incubated at 37° C in a humidified atmosphere of CO_2 in air. Twenty-four h before harvesting, 0 8 µCi of ^{3}H-thymidine was added (The Radiochemical Centre Amersham, England, specific activity 101.7 mCi/mg). The mitogens were added at zero time in saline. The experiments were performed in duplicate or triplicate.

Harvesting of cultures. The cultures were washed twice with cold 0.9 per cent NaCl. Acid-soluble materials were extracted with 2.0 ml 5 per cent trichloracetic acid (TCA) for 1 h at 4° C. The precipitates were washed twice with cold 5 per cent TCA and then dissolved in 0.6 ml Soluene-350 by incubation for 2 h at 50° C. The radioactivity was then counted, in 10 ml toluene based scintillation fluid, in a Packard spectrometer, model A 2425, operated at room temperature

Phytohaemagglutinin. Purified PHA (HA 16) from Wellcome Reagent Ltd., Beckenham, England, was used

Chemical analyses The carbohydrate content was determined with reference to D-glucose by the phenol-H_2SO_4 method (3). The concentration of protein was measured by the method of *Lowry et al* *(14)* with bovine serum albumin as standard.

RESULTS

Isolation of the preparation. Details of the purification are given in Table 1. Haemagglutinating and mitogenic activities were demonstrated in the fractions precipitated from the saline extract by 0–30 and 30–60 per cent saturation of ammonium sulphate. The latter fraction contained several active proteins that could be separated by chromatography on DEAE-Sepharose (Fig. 1). The first peak eluted revealed the highest activities and was purified further on CM-Sepharose (Fig. 2).

SDS gel electrophoresis. The isolated fractions were analysed by SDS polyacrylamide gel electrop-

TABLE 1 *Purification of the* Lathyrus odoratus *Lectin*

Fractions	Yield mg/100 g of seeds	Haemagglutination titre[a]	Mitogenecity
Saline extract	9.000	8	N.D.
Fractions obtained by $(NH_4)_2SO_4$ precipitation			
0–30 %	100	8	+
30–60 %	600	64	+
60–100 %	400	–	N D
Fractions from DEAE-Sepharose			
I	70	64	+
II	90	8	+
III	65	–	N D.
IV	110	–	N D
Fractions from CM-Sepharose			
I A	1	–	N D
I B	30	128	+

[a] Expressed as the reciprocal of the highest dilution of solutions containing 1 mg/ml. showing detectable agglutination Erythrocytes of blood groups A, B and 0 gave similar results

N. D. Not determined

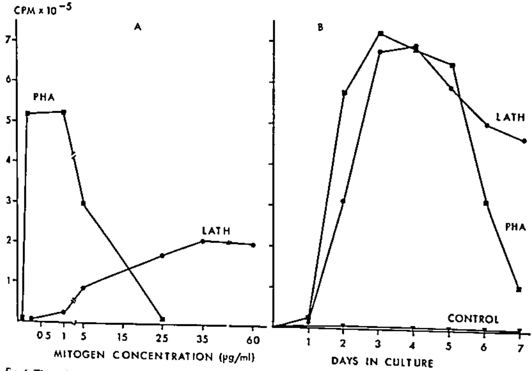


Fig 6 Thymidine incorporation into duplicate cultures seeded with 1.5×10^6 lymphocytes A. Three-day cultures treated with various concentrations of the mitogens B Time course study on cultures stimulated with 1.0 µg/ml of PHA or 35 µg/ml of LATH

DISCUSSION

The present report describes mitogenic activity of a lectin isolated from the seeds of *Lathyrus odoratus* (LATH) by ammonium sulphate fractionation and ion exchange chromatography The purity of the isolated mitogen was tested in two SDS polyacrylamide gel systems The mitogen migrated as a single protein band in the continuous gel electrophoresis However, the discontinuous SDS system revealed in addition a very faint, fast-moving band This confirms the significance of using different gel systems, as pointed out by *Fischetti et al* (4). These results indicate a rather homogeneous lectin However, when the lectin was subjected to isoelectric focusing, multiple bands were obtained Other investigators have reported similar findings with purified lectins (6 15) Multiple bands might conceivably arise from heterogeneity in the sugar chains

The LATH lectin has a molecular weight of 37,000 by gel filtration under neutral conditions. The molecular weight determined by SDS gel electrophoresis was 19,000 These results indicate that the lectin is composed of two sub-units The molecular weight and number of sub-units are similar to those of the mitogenic *Lentil* agglutinin (12) The widely used mitogens PHA and Concanavalin A have molecular weights above 100,000 and four sub-units (11).

The concentration required for optimal stimulation was significantly higher for LATH than for PHA. This may be due to the above-mentioned differences in molecular structure and/or that different lymphocyte populations are involved. The activity of the *Lentil* agglutinin (18) seems to be of the same order as that of LATH, although the comparison is somewhat hampered by differences in the cell culture techniques. In some experiments the lymphocyte stimulation with optimum LATH concentration was equal to that obtained with PHA while in other experiments the optimum LATH dose was less efficient than PHA. This might be due to the fact that lymphocytes from different donors were used in these studies.

It has been shown by other authors that, after the addition of various mitogens, more than 24 h elapse

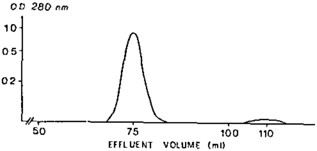


Fig. 4. Gel filtration of fraction I B in Fig. 2 on a column (1.5 × 82 cm) of Bio-Gel P-300 under neutral conditions (0.1 M Tris-HCl pH 8.0). Sample: 13 mg in 2.0 ml. The material in the main peak was eluted corresponding to mol. wt. 37,000 according to a standard curve constructed as described under »Materials and Methods«. Fractions of 2 ml were collected at a flow rate of 4 ml/h.

Isoelectric focusing. The lectin formed five peaks (Fig. 5). Haemagglutinating and mitogenic activities were found in the materials eluted in the four major peaks, which focused between pI 5.9 and 7.5.

Carbohydrate content. The neutral sugar content was 0.5 per cent, calculated as D-glucose.

Haemagglutinating activity. The LATH lectin and PHA gave similar titres when starting at a concentration of 1 mg/ml.

Mitogenic activity. The relationship between ^{3}H-thymidine incorporation and the number of lymphocytes per culture was examined. For both PHA and LATH, three was a linear relationship within the range of cells tested ($0.25 - 2 \times 10^6$/culture).

On exposure of lymphocytes to the LATH mitogen, a strong dose dependent stimulation of the incorporation of labelled thymidine was found (Fig. 6 A). A plateau of maximum stimulation was observed when the concentration was raised to 35 µg/ml and higher. The concentration of PHA required for maximum incorporation was 0.25–1.0 µg/ml. When an optimal amount of mitogen was added to each culture and the incorporation of labelled thymidine was measured at different times, a significant increase could be detected for both LATH and PHA after 24 h (Fig. 6 B). The DNA synthesis was maximal between the second and fifth day for PHA and between the third and fifth day for LATH.

Cells from two different donors were used in the two experiments depicted in Figs 6 A & B. The maximum amounts of incorporation were similar for PHA and LATH for one subject. With cells from the other donor, LATH was clearly less effective than PHA. The results of further experiments with different subjects were in accordance with these findings (not depicted). The mean coefficient of variation for stimulation experiments was 2.4%.

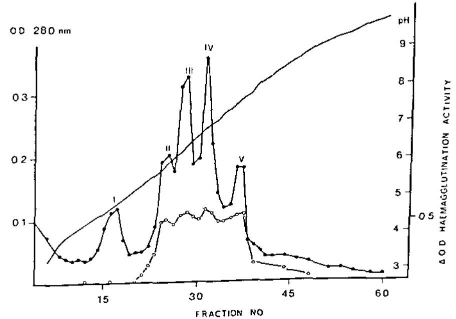


Fig. 5. Isoelectric focusing of the material from peak I B in Fig. 2 after dialysis against saline. The focusing was performed in a LKB 8101 column with 2.5 per cent Ampholine in sucrose according to the manufacturer's instructions. Fractions of 1.5 ml were collected, dialyzed against saline and assayed for haemagglutinating activity.

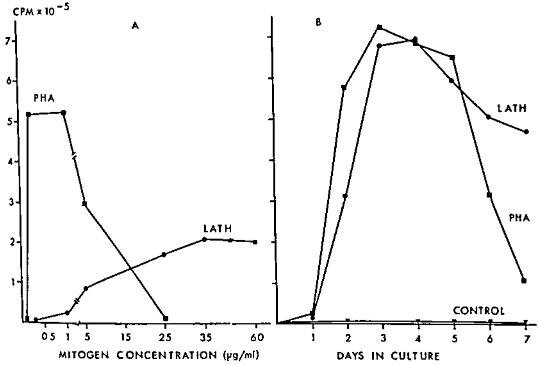


Fig 6 Thymidine incorporation into duplicate cultures seeded with 1 5 × 10^6 lymphocytes A Three-day cultures treated with various concentrations of the mitogens B Time course study on cultures stimulated with 1.0 μg/ml of PHA or 35 μg/ml of LATH

DISCUSSION

The present report describes mitogenic activity of a lectin isolated from the seeds of *Lathyrus odoratus* (LATH) by ammonium sulphate fractionation and ion exchange chromatography The purity of the isolated mitogen was tested in two SDS polyacrylamide gel systems The mitogen migrated as a single protein band in the continuous gel electrophoresis However the discontinuous SDS system revealed in addition a very faint, fast-moving band This confirms the significance of using different gel systems as pointed out by *Fischetti et al* (4) These results indicate a rather homogeneous lectin However when the lectin was subjected to isoelectric focusing, multiple bands were obtained Other investigators have reported similar findings with purified lectins (6, 15) Multiple bands might conceivably arise from heterogeneity in the sugar chains

The LATH lectin has a molecular weight of 37,000 by gel filtration under neutral conditions The molecular weight determined by SDS gel electrophoresis was 19,000 These results indicate that the lectin is composed of two sub-units The molecular weight and number of sub-units are similar to those of the mitogenic *Lentil* agglutinin (12) The widely used mitogens PHA and Concanavalin A have molecular weights above 100,000 and four sub-units (11).

The concentration required for optimal stimulation was significantly higher for LATH than for PHA This may be due to the above-mentioned differences in molecular structure and/or that different lymphocyte populations are involved. The activity of the *Lentil* agglutinin (18) seems to be of the same order as that of LATH, although the comparison is somewhat hampered by differences in the cell culture techniques. In some experiments the lymphocyte stimulation with optimum LATH concentration was equal to that obtained with PHA while in other experiments the optimum LATH dose was less efficient than PHA This might be due to the fact that lymphocytes from different donors were used in these studies.

It has been shown by other authors that, after the addition of various mitogens, more than 24 h elapse

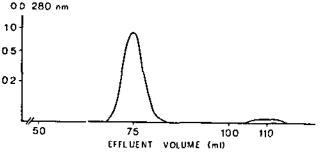


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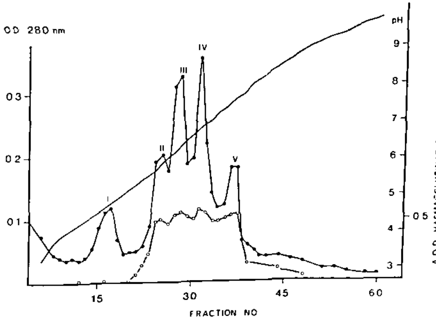


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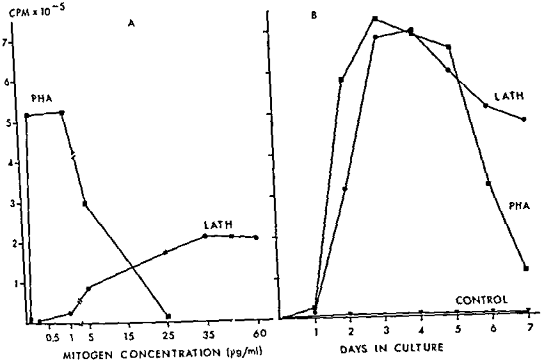


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It has been shown by other authors that, after the addition of various mitogens, more than 24 h elapse

before any increase in DNA synthesis is seen (2, 7). Similar results were found with the LATH lectin.

The LATH lectin contained 0.5 per cent carbohydrates. Other purified lectins are reported to contain 2–10 per cent (16).

The author wishes to thank Dr. *R. Andersen* for performing the isoelectric focusing and Dr. *L. O. Frøholm* & Dr. *L. Haaheim* for the analyses on SDS gel electrophorsis.

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EFFECT OF HALOTHANE ANAESTHESIA ON SECONDARY ANTIBODY RESPONSE AND MITOGEN-INDUCED LYMPHOCYTE TRANSFORMATION IN THE CHICKEN

M SALO, O LASSILA, M VILJANEN, J ESKOLA and L KANGAS

Departments of Anesthesiology, Medical Microbiology and Pharmacology, University of Turku, Turku, Finland

Salo, M, Lassila, O., Viljanen, M, Eskola, J & Kangas, L. Effect of halothane anaesthesia on secondary antibody response and mitogen-induced lymphocyte transformation in the chicken. Acta path microbiol scand Sect C, 86 105–108, 1978

The effect of a single halothane anaesthesia on the secondary antibody response and the lymphocyte response *in vitro* to phytohaemagglutinin (PHA) and concanavalin A (Con A) was studied. The antigens used were bovine serum albumin (BSA) and killed *Brucella abortus* organisms *(Brucella)*. IgG and IgM antibodies against these antigens were quantified by solid-phase radioimmunoassay Lymphocyte transformation was studied by a whole blood micromethod The chickens were anaesthetised for two hours with 2% v/v halothane The halothane concentration of the blood was measured by gas chromatography at the end of the anaesthesia

Halothane anaesthesia had no effect on the secondary antibody response against BSA and *Brucella* In contrast, it caused a significant decrease in the lymphocyte response to PHA and Con A This suppression was transient, however, and full recovery of the lymphocyte function was observed by the first day after anaesthesia

Key words Antibody formation, halothane, lymphocyte transformation.

M Salo, Department of Anesthesiology, University Central Hospital, SF-20520 Turku 52, Finland

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Halothane is known to affect immune reactivity in many ways A long exposure to halothane depresses bone marrow function and decreases peripheral granulocyte and lymphocyte counts (7) Contradictory observations exist as regards the interference of halothane with chemotaxis *in vitro* (10, 12, 18) *In vivo* halothane is reported to decrease the mobilisation of neutrophils to peritoneal exudate (4,5) The phagocytic functions of both circulating (5) and fixed phagocytes (17) are also affected Observations on the effects of halothane on B-cell functions are contradictory. It decreases the number of antibody-forming cells in the spleen (13, 14, 27, 28), but it is reported to have no effect on agglutinin titres either in the primary or secondary antibody responses (14, 24, 28) The effects on T-cell functions have been studied by exposing the lymphocytes to halothane both *in vitro* and *in vivo*. Halothane mixed with the culture medium of lymphocytes inhibits mitogenic (6, 9) and cytotoxic (8) responses of lymphocytes in a dose-dependent manner. Repeated halothane anaesthesia suppresses the response of chicken splenic lymphocytes to concanavalin A (Con A) (24)

Monitoring the concentrations of class-specific antibodies by radioimmunoassay may give more accurate information than the agglutination method as regards the effect of halothane anaesthesia on antibody production. More exact kinetic studies of the mitogenic responses of lymphocytes are also possible with the new whole blood micromethod (16), since the animals need not be killed. Therefore, the present work was undertaken to clarify the

effect of halothane anaesthesia on the kinetics of secondary antibody production against a T-dependent and a T-independent antigen and mitogen-induced lymphocyte transformation.

MATERIAL AND METHODS

Animals

The experimental animals were four-week-old line P White Leghorn chickens, homozygous for the major histocompatibility locus, genotype B^2B^2.

Anaesthesia

The chickens were anaesthetised for two hours with 2% v/v halothane in a mixture of 30% oxygen and 70% nitrogen at a gas flow rate of 3 l/min, using the Drager-Vapor® vaporiser (Dragerwerk Lubeck, Lubeck, Germany) In preliminary experiments, 1% v/v concentration of halothane in the gas mixture inspired gave only light anaesthesia in the chicken In the present work with 2% v/v halothane, a proper third-stage surgical anaesthesia was achieved. The halothane concentration of the blood was measured at the end of the anaesthesia by gas chromatography (15). In the box where the chickens were anaesthetised, the oxygen percentage was 22–25% measured by a Beckman Oxygen Analyser, Model D2 (Beckman Instruments Ltd, Glennrothes Fife, Scotland) No CO_2 could be measured in this box by a capnograph (Godart NV, Bilthoven, England)

To check the effect of the experimental arrangement on the pH, PCO_2 and PO_2 and on the rectal temperature, five additional chickens anaesthetised with 2% v/v halothane for two hours as before and five non-anaesthetised controls were studied Blood samples were taken into capillary tubes at the end of anaesthesia from the wing artery and from the anterior metatarsus by removing some scales The pH of these blood samples was measured by a K 172 calomel cell and G 299 A glass electrode (Radiometer A/S, Copenhagen, Denmark), PCO_2 by a PCO_2 electrode type E 5036 (Radiometer A/S, Copenhagen, Denmark) and PO_2 by a PO_2 electrode type E 5046 (Radiometer A/S, Copenhagen, Denmark), all at 37° C The rectal temperatures were measured by an electric thermometer (Thermorapid Medeor G.m b H., Hamburg, Germany)

Secondary Antibody Response

Seventeen chickens were immunised intraperitoneally with 5 mg bovine serum albumin (BSA, Fraction V, Armour Pharmaceutical Co. Ltd, Eastbourne, England) and 10^7 formalin-killed *Brucella abortus* organisms (*Brucella*) Nine animals were anaesthetised eight days after the primary immunisation and eight chickens served as a non-anaesthetised control group The chickens were given a booster of the same size as the primary immunisation 15 minutes after the onset of the anaesthesia.

Blood samples were drawn for antibody titrations one day before and one, 3, 6 and 10 days after the anaesthesia IgG and IgM antibodies to BSA and *Brucella* were quantified by solid-phase radioimmunoassay (23).

Lymphocyte Response to Mitogens

The mitogenic responses of lymphocytes were studied in 10 anaesthetised chickens and 10 non-anaesthetised controls one day before and one hour, one, 3, 6 and 10 days after the anaesthesia by a whole blood micromethod (16). The mitogens used were phytohaemagglutinin (PHA P, Difco Laboratories, Detroit, Mich , USA) in a final concentration of 1200 μg/ml and Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) in a final concentration of 20 μg/ml The mitogenic responses were determined by the incorporation of [^{125}I]-5-iodo-2-deoxyuridine ([^{125}I] UdR, specific activity 90–110 mCi/mg, The Radiochemical Centre, Amersham, England)

Statistics

Student's *t*-test was used to compare the arithmetic means of comparable data.

RESULTS

pH, PCO_2, PO_2, Rectal Temperature and Blood Halothane Concentration at the End of Anaesthesia

The pH, PCO_2 and PO_2 values are given in Table I. The rectal temperature at the end of anaesthesia was 35 4

TABLE I *pH, PCO_2 and PO_2 after Two Hours' Anaesthesia with 2% v/v Halothane in Five Anaesthetised and Five Non-anaesthetised Control Chickens Means ± s d*

	pH	PCO_2 (mmHg)	PO_2 (mmHg)
Samples from the anterior metatarsus capillaries			
Halothane group	7 383 ± 0 069[b] (n = 4)	30.3 ± 4 0[a] (n = 3)	63 8 ± 5 4 (n = 4)
Controls	7 540 ± 0 061	23 2 ± 3.6	60 0 ± 5 4
Samples from the wing artery			
Halothane group	7 486 ± 0.036	26 0 ± 5.6 (n = 3)	84 0 ± 9 5[a] (n = 4)
Controls	7 537 ± 0 095	20.0 ± 1.4 (n = 2)	67 3 ± 9 0 (n = 4)

a) = $P<0.05$
b) = $P<0.01$

TABLE 2 *Secondary Antibody Responses to BSA and* Brucella *after Two Hours' Anaesthesia with 2% v/v Halothane on Day 0 in Nine Anaesthetised Chickens and Eight Non-anaesthetised Controls Primary Immunisation carried out on Day − 8 Antibody Concentrations Indicated as Percentage of Pooled Standard Plasma Means ± s d*

Quantification	Groups	Days after halothane anaesthesia				
		− 1	+ 1	+ 3	+ 6	+ 10
IgG-anti-BSA	Halothane	55.8 ± 44.6	5.8 ± 11.8	36.7 ± 41.3	62.8 ± 44.8	28.8 ± 31.7
	Control	46.2 ± 57.9	17.2 ± 42.6	46.8 ± 62.1	108.1 ± 60.9	58.3 ± 51.5
IgM-anti-BSA	Halothane	53.5 ± 47.1	11.7 ± 12.3	41.1 ± 25.9	52.2 ± 68.8	17.1 ± 14.2
	Control	50.8 ± 72.1	13.4 ± 16.1	50.5 ± 38.0	63.5 ± 73.0	18.8 ± 24.4
IgG-anti-*Brucella*	Halothane	15.2 ± 25.9	58.5 ± 58.0	76.7 ± 80.6	131.1 ± 94.5	250.0 ± 127.8
	Control	23.0 ± 31.6	46.9 ± 57.4	114.2 ± 66.1	91.0 ± 57.8	236.5 ± 91.8
IgM-anti-*Brucella*	Halothane	13.1 ± 38.8	26.7 ± 73.8	27.6 ± 32.9	73.0 ± 47.3	64.1 ± 68.2
	Control	0.5 ± 0.4	1.4 ± 1.9	32.7 ± 30.9	83.3 ± 48.3	77.8 ± 53.9

± 1.2° C (mean ± s d) in the anaesthetised chickens and 41.2 ± 0.13° C in the non-anaesthetised control chickens (P < 0.001) The blood halothane concentration at the end of anaesthesia was 0.221 ± 0.054 g/l in the antibody titration group and 0.236 ± 0.038 g/l in the lymphocyte stimulation group

Secondary Antibody Response

There were no significant differences in the IgG- and IgM-anti-BSA and IgG- and IgM-anti-*Brucella* concentrations between the anaesthetised and the non anaesthetised chickens during the follow-up of 10 days after anaesthesia (Table 2)

Lymphocyte Response to Mitogens

A slight decrease in spontaneous incorporation of [125I]UdR was observed one hour after halothane anaesthesia (Fig 1), but at the same time the PHA- and Con A-induced transformation of peripheral blood lymphocytes decreased to 43 and 23% of the control group values, respectively This suppression of mitogenic responses was totally restored by the first day after anaesthesia No rebound effect was observed

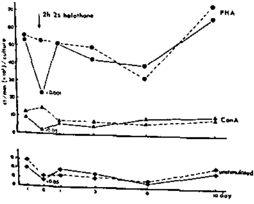


Fig 1 In vitro lymphocyte responses to PHA and Con A in 10 anaesthetised chickens (—) and in 10 non-anaesthetised controls (----) The mitogen responses on day 0 were measured one hour after the end of

DISCUSSION

The effect of halothane on immune responses in the chicken was measured using an anaesthesia resembling a conventional clinical anaesthesia Secondary immunisation during anaesthesia was selected for the model because subjects exposed to earlier contact with many antigens can be expected to have a new contact with some of these antigens during the operation The antigens BSA and *Brucella* represent T-dependent (1, 3) and T-independent antigens (22, 26), respectively The mitogens PHA and Con A activate T-lymphocytes selectively in the chicken (2, 21) and are commonly used to evaluate cell-mediated immunity

Two hours' anaesthesia of the chicken with 2% v/v halothane in this study did not affect the secondary antibody response against BSA and *Brucella* when the class-specific antibody concentrations were measured by radioimmunoassay This verifies the results obtained with the agglutination method (24) Halothane impairs the function of antibody-producing splenic cells (13, 14, 27, 28), but the effect is very transient (27) The functional reserves of antibody-producing cells may be so large that the decrease is not manifested in antibody concentrations In addition, simultaneous changes possibly occurring in the activity of regulatory T-cells may compensate a temporary deficiency of B-cells during halothane anaesthesia

The impairment of the mitogenic responses of peripheral blood lymphocytes to PHA and Con A observed in this study after halothane anaesthesia was of short duration This finding concurs with the inhibition of mitogenic lymphocyte responses (6, 9) and the inhibition of cell-mediated cytotoxicity (8) with halothane added *in vitro*, as well as with the inhibition of antibody-dependent cellular cytotoxicity by halothane *in*

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	pH	PCO_2(mmHg)	PO_2(mmHg)
Samples from the anterior metatarsus capillaries			
Halothane group	7 383 ± 0 069[b] (n = 4)	30.3 ± 4.0[a] (n = 3)	63 8 ± 5.4 (n = 4)
Controls	7 540 ± 0.061	23 2 ± 3 6	60 0 ± 5 4
Samples from the wing artery			
Halothane group	7 486 ± 0 036	26 0 ± 5.6 (n = 3)	84.0 ± 9.5[a] (n = 4)
Controls	7.537 ± 0.095	20 0 ± 1 4 (n = 2)	67.3 ± 9.0 (n = 4)

a) = $P < 0\ 05$
b) = $P < 0\ 01$

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CROSSED IMMUNOELECTROPHORESIS AND ELECTROIMMUNOASSAY OF HUMAN IgG SUBCLASSES

VIVI-ANNE OXELIUS

Department of Pediatrics and Institute of Medical Microbiology, University of Lund, S-221 85 Lund, Sweden

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V.-A. Oxelius, Department of Pediatrics, University of Lund, S-221 85 Lund, Sweden

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This paper reports 1) qualitative data of IgG

subclasses in normal serum pools, Cohn Fraction II and individual sera by crossed immunoelectrophoresis, 2) quantitative data about normal serum pools, Cohn Fraction II and individual sera by electroimmunoassay and 3) a comparison of these data with Gm factors representative of the subclasses of IgG1 and IgG3. The sera were therefore typed for the genetic markers Gm(1), Gm(4) and Gm(5). In the immunochemical studies rabbit antisera were used.

MATERIALS AND METHODS

1 *Normal Human Sera and Myeloma Proteins*

A normal serum pool (IgG content 128 I.U./ml) prepared from blood somples from 400 registered blood donors was used.

Reference serum pool 67/97 (IgG content 96 I.U./ml) from WHO International Reference Centre for Immunoglobulins, Lausanne, Switzerland.

Individual sera from 20 normal adults and different Gm patterns were analysed

Cohn Fraction II(Kabi 64) was kindly supplied by Kabi AB, Sweden.

IgG myeloma proteins of all four IgG subclasses were isolated from sera of patients with multiple myeloma by ammonium sulphate precipitation, preparative electrophoresis in agarose, DEAE chromatography (*Peterson & Sober* 1956) and Sephadex G 200 gel filtration (*Flodin & Killander* 1962). The IgG myeloma proteins were classified by Gm typing or by testing them with immunodiffusion and immunoelectrophoresis against specific subclass antisera. For reference purpose anti-IgG1, anti-IgG2 and anti-IgG4 were kindly placed at our disposal by Dr. *Skvaril*, and anti-IgG3 by Dr. *Grey*.

2. *IgG Subclass Antisera*

Antisera were prepared in rabbits The method of *Spiegelberg & Weigle* (1968) was followed. The rabbits were made tolerant to three of four IgG subclasses given by intravenous injection of 15–20 mg of a centrifuged solution of a mixture of isolated myeloma protein Simultaneously the immunizing dose of a 3–5 mg solution of *myeloma protein in Freund's complete* adjuvant was given subcutaneously Booster doses of the immunizing IgG subclass were given once a week 3 or 4 times Antisera suitable for use in the immunodiffusion assay were obtained 7–12 weeks after the first immunizing dose had been given Judging from our experience, IgG subclass specific antibodies in rabbits are detected already early in the course of immunization Repeated tolerance doses or immunizing doses did not improve the result High-titered antisera against IgG3 were readily produced by rabbits without previous preparation of the animals with tolerant doses of the other IgG subclasses.

When necessary antisera were rendered specific for IgG by *absorption* with serum fractions or isolated serum proteins by incubation at 37° C for one hour and at 4°C overnight. Subclass specificity was secured by absorption in the same way but with isolated *myeloma* proteins including all heavy-light chain combinations and IgG subclasses with different *Gm markers*. Also IgG subclass deficient sera were used.

3. *Quality and quantity of IgG Subclasses*

Crossed immunoelectrophoresis was performed according to *Laurell* (1965) and *Ganrot* (1972). Barbital buffer, 0.75 M, pH 8.6, containing 0 002 M Ca^{++} was used. In the first step sera were run undiluted and Cohn Fraction II 20 g/l. Antiserum concentration was chosen 5–20%.

Immunoelectrophoresis was run as described by *Ouchterlony* (1962), in agarose.

Electroimmunoassay was done in the way described by *Laurell* (1972) for other proteins. The values obtained were calculated relative to the concentration in the reference serum pools. The concentrations of IgG subclasses in the WHO reference serum pool 67/97 were given as: 5 1 g/l for IgG1, 2.5 g/l for IgG2, 0 55 g/l for IgG3 and 0.35 g/l for IgG4 (*Steinberg et al* 1973) The values in this article were given according to the values in this reference serum (*Steinberg et al.* 1973) Barbital buffer, 0.75 M, pH 8.6, containing 0 002 M Ca^{++} was used as electrophoresis buffer. Samples were diluted 1:10, 1:8, 1:2 and 1.2 in buffer solution for determination of IgG1, IgG2, IgG3 and IgG4, respectively. The holes in the agarose plate were placed in appropriate positions for the IgG subclass analysed. The lowest possible antiserum concentration (4–11%) was chosen The electrophoresis was run 4 hours for IgG1 and IgG3 and 3 hours for IgG2 and IgG4 at 10 Vper cm Agarose from Miles-Seravac, Maidenhead, was used in all the experiments. After the plates had dried they were stained with Comassie Brilliant Blue R-250 (Mann Research Laboratory, N.Y., USA) Double determinations were made of 20 samples from registered blood donors and the interplate reproducibility of the test was calculated for each IgG subclass

Sera frozen and thawed up to five times, and stored at room temperature for 1, 3 and 5 days were compared with fresh serum for any difference in the results of the electroimmunoassay.

Single radial immunodiffusion according to *Mancini et al.* (1965).

Gm determination as described by *Grubb* (1956) *adapted in microtiter system* (*Borel* 1967).

RESULTS

IgG1. On crossed immunoelectrophoresis (Fig. 1) and electroimmunoassay (Fig. 7) the IgG1 of the WHO pool 67/97 as well as of our own standard serum pool gave a precipitation pattern anodal and cathodal to the application hole. With these methods deficient antisera were easily recognized. An antiserum against Gm(1) myeloma protein produced strong precipitates with sera from individuals homozygous with Gm(1) type while sera from Gm(4) homozygous individuals showed thinner and more diffuse precipitation lines with the same

antiserum Antisera against both Gm(1) and Gm(4) were therefore used. IgG1 of both Gm(1) and Gm(4) homozygous individuals spread over the same electrophoretic area (Fig. 2, Fig. 7) showed roughly the same concentrations. The amount of IgG1 in Cohn Fraction II (10 g/l) was about 50 per cent of that in the WHO pool 67/97 (Table 1). The anodal portion of Cohn Fraction II was relatively smaller than that for the WHO serum pool (Fig. 1, Fig. 7). The mean value, standard deviation and range for 20 blood donors are given in Table 2. There was good agreement of the IgG1 values found with electroimmunoassay and single radial immunodiffusion (r = 0 97)

IgG2 On crossed immunoelectrophoresis (Fig 3) and electroimmunoassay (Fig. 8) the IgG2 of the

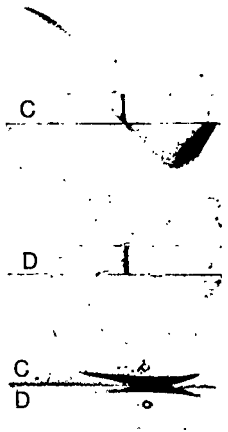


Fig 2 Crossed immunoelectrophoresis and immunoelectrophoresis of one homozygous Gm(1) serum (C) and one homozygous Gm(4) serum (D) against anti IgG1 antiserum Anode is upwards and to the left.

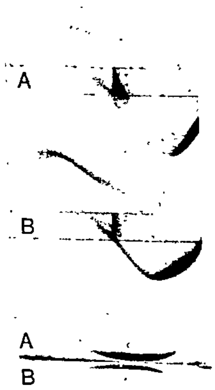


Fig 1 Crossed immunoelectrophoresis and immunoelectrophoresis of Cohn Fraction II (20 g/l) (A) and WHO pool 67/97 (B) against anti IgG1 antiserum Anode is upwards and to the left

TABLE 1 *IgG Subclass Levels in Cohn Fraction II*

	IgG1	IgG2	IgG3	IgG4
		g/l		
WHO pool 67/97[a]	5.10	2 50	0 55	0 35
Cohn Fraction II 10 g/l	2 55	1.25	0.59	0 04

[a] According to the levels determined by *Steinberg et al.* 1973

TABLE 2. *IgG Subclass Levels in g/l in 20 Healthy Adults*

	IgG1	IgG2	IgG3	IgG4[a]
Mean	7.55	3.80	0.72	0.54[b]
S.D.	2.52	1.53	0.27	
Range	4.22–12.92	1.17–7.46	0.41–1.28	<0.01–2.65

a) Amounts less than 0.01 g/l regarded as 0.005 g/l.
b) Non-detectable IgG4 values (less than 0.01 g/l) were found in 25%.

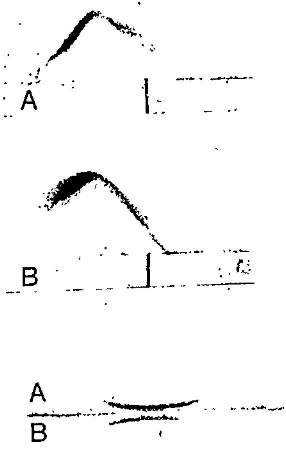


Fig 3 Crossed immunoelectrophoresis and immunoelectrophoresis of Cohn Fraction II (20 g/l) (A) and WHO pool 67/97 (B) against anti IgG2 antiserum. Anode is upwards and to the left.

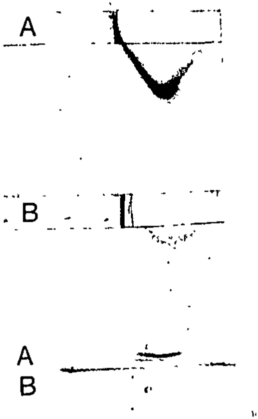


Fig 4. Crossed immunoelectrophoresis and immunoelectrophoresis of Cohn Fraction II (20 g/l) (A) and WHO pool 67/97 (B) against anti IgG3 antiserum. Anode is upwards and to the left.

standard pool and individual sera gave a precipitation pattern both anodal and cathodal to the application hole. An antiserum raised by only one IgG2 myeloma protein sometimes gave incomplete »open« precipitation lines with some serum samples. Therefore antiserum prepared from two or more IgG2 myeloma proteins of different electrophoretic mobility was used. The amount of IgG2 in Cohn Fraction II (10 g/l) was about 50 per cent of that in the WHO pool (Table 1). The anodal part of Cohn Fraction II was relatively smaller than that of the WHO pool (Fig 3, Fig. 8) The mean value, standard deviation and range of samples from 20 blood donors are given in Table 1 There was good agreement between the IgG2 values found with the electroimmunoassay and with the single radial immunodiffusion (r = 0.97).

IgG3 The IgG3 of the standard serum pool, Cohn Fraction II and individual sera were situated cathodally in the crossed immunoelectrophoretic pattern (Fig 4) and in the electroimmunoassay (Fig. 9) Sera from individuals homozygous to Gm(5) gave a precipitation line which spread over the whole cathodal area, while sera from individuals lacking Gm(5) showed only a short precipitate in the cathodal area (Fig. 5, Fig. 9) Individuals lacking Gm(5) showed low concentrations The amount of IgG3 in Cohn Fraction II (10 g/l) was about the same as that of the WHO pool. The mean value, standard deviation and range of IgG3 are given in Table 1. The correlation coefficient between values determined by electroimmunoassay and single radial immunodiffusion was 0 93.

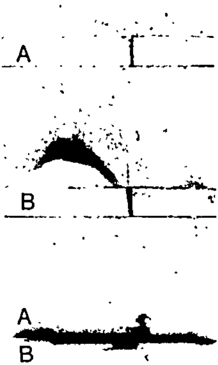


Fig 6 Crossed immunoelectrophoresis and immunoelectrophoresis of Cohn Fraction II (20 g/l (A) and WHO pool 67/97 (B) against anti IgG4 antiserum Anode is upwards and to the left.



Fig 5 Crossed immunoelectrophoresis and immunoelectrophoresis of one homozygous Gm(-5) serum (E) and one homozygous Gm(5) serum (F) against anti IgG3 antiserum Anode is upwards and to the left.

IgG4 The IgG4 of the standard serum pool, Cohn Fraction II and individual sera were situated mainly anodally in crossed immunoelectrophoresis (Fig 6) and electroimmunoassay (Fig. 10). In Cohn Fraction II (10 g/l) 10 per cent of that in the WHO pool was found (Fig 6, Fig. 10) The mean value and range for 20 blood donors are given in Table 1. The concentrations ranged from not detectable with this method to relatively high values. There was good agreement with the values found with the electroimmunoassay and the single radial immunodiffusion technique (r = 0.99)

Double determination of 20 sera was carried out to assess the reproducibility of the method. The determinations were made on different plates to assess the interplate variation. The variation coefficients of the interplate determinations were 3.4%

TABLE 2. *IgG Subclass Levels in g/l in 20 Healthy Adults*

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S.D.	2.52	1.53	0.27	
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a) Amounts less than 0.01 g/l regarded as 0.005 g/l
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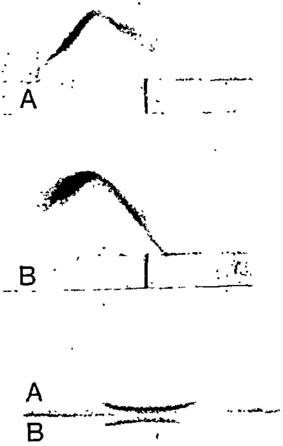


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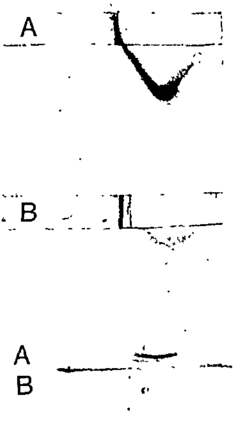


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DISCUSSION

The electroimmunoassay was found to be most convenient for quantitation of human IgG subclasses. This method utilizes the differences in electrophoretic mobility of the IgG subclasses. In assays using agarose gel, barbital buffer pH 8.6 with Ca^{++} the IgG1 and IgG2 molecules migrated to both the anode and the cathode. The IgG3 molecules migrated only to the cathode and the IgG4 molecules only to the anode. Also antisera containing antibodies to more than one IgG subclass could be used in this method. For an antiserum with specificity of both IgG3 and IgG4 could be used in measuring the anodal precipitate as IgG4 and the cathodal precipitate as IgG3.

The mean, S.D. and normal range of IgG1, IgG2, IgG3 and IgG4 were generally in agreement with the results reported by other authors (*Steinberg et al.* 1973). For IgG4 which is the smallest of the IgG subclasses the range of the values found was wider than for the other IgGsubclasses. In 5 out of 20 sera IgG4 was not detected (< 0.01 g/l) studied with this method.

Sera from individuals homozygous for Gm(5) showed long distinct precipitates in electroimmunoassay, while sera from individuals lacking Gm(5) showed short precipitates. It confirmed earlier findings that Gm(5,5) sera have the highest IgG3 concentrations (*Yount et al.* 1967). Gm(1,1) and Gm (4,4) sera also representing different allelles on the other hand showed IgG1 precipitates that distended over the same electrophoretic area and showed roughly the same concentrations of IgG1. Electroimmunoassay and crossed immunoelectrophoresis were useful methods to evaluate the conditions of the IgG subclass antisera (*Grubb* 1972). Sometimes individual sera gave incomplete, diffuse or thin precipitates with some antisera in electroimmunoassay and crossed immunoelectrophoresis. It was found that immunization with only one myeloma protein often resulted in such deficient antisera. *Immunization with two or more myeloma* proteins with different electrophoretic mobility and different Gm markers within the same IgG subclass gave better quality of IgG subclass antisera.

In Cohn Fraction II the relative amounts of IgG1, IgG2 and IgG4 were lower than those found in normal serum pools (Table 1). There was also another electrophoretic distribution of IgG subclasses in Cohn Fraction II. The conclusion is that Cohn Fraction II does not represent the whole spectrum of IgG subclass molecules compared to a normal serum pool. Cohn Fraction II could not be used as standard in IgG subclass determinations. The deficiency of IgG subclasses in Cohn Fraction II should be considered also in other tests.

I thank Mrs *Ann-Margreth Carlsson* for skilful technical assistance.

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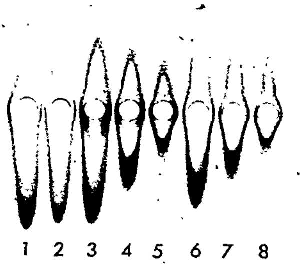


Fig. 7. Quantitation of IgG1 in electroimmunoassay of Gm(1,1) serum 1:10 (1), Gm(4,4) serum 1:10(2), Cohn Fraction II (20 g/l) 1:10(3), 1:20(4), 1.40(5) and the WHO pool 67/97 1:10(6), 1: 20(7), 1:40(8).



Fig. 8. Quantitation of IgG2 in electroimmunoassay of Cohn Fraction II (20 g/l) 1.8(1), 1.16(2), 1:32(3) and the WHO pool 67/97 1:8(4), 1 16(5), 1:32(6)



Fig 9 Quantitation of IgG3 by electroimmunoassay of Gm(−5,5) serum 1.2(1), Gm(5,5) serum 1:2(2), Cohn Fraction *II (20 g/l)* 1.2(3), 1 4(4), 1:8(5) and the *WHO* pool 67/97 1·2(6), 1 4(7), 1 8(8)

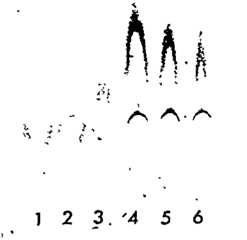


Fig 10. Quantitation of IgG4 by electroimmunoassay of Cohn Fraction II (20 g/l) 1 2(1), 1 4(2), 1 8(3) and the *WHO pool 67/97 1 2(4), 1 4(5), 1 8(6)*

for IgG1, 5.5% for IgG2, 5.1% for IgG3 and 1.5% for IgG4. Storage of serum at room temperature for 1, 3 and 5 days and repeated freezing and thawing of sera up to five times before analysis did not appreciably affect the results of quantitation of any of the IgG subclasses tested.

DISCUSSION

The electroimmunoassay was found to be most convenient for quantitation of human IgG subclasses. This method utilizes the differences in electrophoretic mobility of the IgG subclasses. In assays using agarose gel, barbital buffer pH 8.6 with Ca^{++} the IgG1 and IgG2 molecules migrated to both the anode and the cathode. The IgG3 molecules migrated only to the cathode and the IgG4 molecules only to the anode. Also antisera containing antibodies to more than one IgG subclass could be used in this method. For an antiserum with specificity of both IgG3 and IgG4 could be used in measuring the anodal precipitate as IgG4 and the cathodal precipitate as IgG3.

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IN VITRO LOCOMOTION OF HUMAN LEUKOCYTES

Photographically Determined Distribution in Millipore Filters at Different Incubation Times

AKSEL SCHREINER

Medical Department B, Faculty of Medicine, University of Bergen, N-5016 Haukeland sykehus, Bergen, Norway

Schreiner A In vitro locomotion of human leukocytes Photographically determined distribution in millipore filters at different incubation times Acta path microbiol scand Sect C, 86 117-121, 1978

The distribution of leukocytes in Boyden millipore filters after stimulation with casein was determined after 30 and 180 min by the use of a photographic method After 30 min the distribution was characterized by a second degree curve with a falling slope and after 180 min by a linear curve with a rising slope The results showed that with most of the modifications of the Boyden method in use at present, the choice of incubation time is crucial for the evaluation of counting results

Key words Chemotaxis measurement of, leukocytes, human, locomotion

A Schreiner, Med Dept B, N-5016 Haukeland sykehus, Norway

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Boyden's filter technique (*Boyden* 1962) still seems to be the most attractive for measuring chemotaxis The greatest advantage of this method lies in its simplicity and in the possibility to change cell media and concentration gradients at will However, statistical studies of the reproducibility are still scanty Determination of chemotactic response using the millipore filter method is made according to at least three principles One possibility is to count the cells that have passed the entire depth of the filter after a given time (Distal surface counting, *Boyden* 1962, double filter techniques, *Keller & al* 1972, *Gallin & al* 1973) Another method implies counting of cells that are present at a given time at a given level, *i e* in a given section within the filter (In-filter counting, *Zigmond & Hirsch* 1973 *Wilkinson* 1974) Finally, the distance travelled by the fastest moving cells may be measured (Leading front method, *Zigmond & Hirsch* 1973, *Wilkinson* 1974)

All these methods offer partial information on the kinetics of a cell population, the information being obviously time-dependent To investigate this influence of incubation time, the leukocyte distribution in Boyden filters after chemotactic stimulation with casein was determined by in-filter counting after two different periods of incubation To serve this purpose, a photographic cell-counting method was developed

MATERIALS AND METHODS

All glassware was siliconized Micropipettes and vials were washed with ethanol and rinsed with tapwater *Chemical solutions* Dextran T 500 was purchased from Pharmacia Fine Chemical, Uppsala, Sweden, Heparin 500 i u per ml from A/S Apothekernes Laboratorium, Oslo, Norway and Casein Hammarsten from Merck, Darmstadt, West Germany All reagents were analytical grade

Isolation of leukocytes Each ml of heparinized (10 i u per ml) blood was mixed with 0 2 ml 4 5 w/v per cent Dextran T 500 and the blood cells were allowed to settle in plastic tubes for 45 min at room temperature The leukocyte-rich supernatant was removed and centrifuged at 600 × g for 5 min To lyse erythrocytes, the sediment was resuspended in 8 g per litre ammonium chloride, and after 3 min centrifuged at 300 × g for 5 min The cell pellet was washed in physiological NaCl, centrifuged at 300 × g for 5 min and resuspended in Hanks' balanced salt solution The cell count was adjusted to 0 25 × 10⁷ cells per ml and the pH to 7 4 The leukocyte suspension contained from 55 to 66 per cent granulocytes

Chemotaxis. Modified Boyden chambers from Neuroprobe, Bethesda, Maryland, U.S.A., and nitrocellulose filters from Millipore Filter Corp., Bedford, Massachusetts, U.S.A. (SSWPO 1300 lot 129281) with a pore size of 3 µm, were used in all experiments Leukocytes were applied to the filters by centrifugation of 0.4 ml cell suspension in a Shandon Elliot cytocentrifuge at 500 × *g* for 3 min. Each filter was rapidly transferred to a chamber, the lower compartment of which had been filled with a mixture of 80 per cent saturated casein in Hanks' solution (appr. 4 mg per ml) and 20 per cent pooled, heat activated (37° C for 15 min) human serum The upper compartment was filled with 2 per cent human serum albumin in Hanks' solution, and the chambers were incubated at 37° C at 100 per cent relative moisture. After the incubation period (*vide infra*), the filters were carefully transferred to a staining rack, fixed in a mixture of 10 per cent methanol in absolute ethanol, dehydrated in increasing concentrations of ethanol, stained with haematoxylin, made optically homogenous with xylene and mounted on glass slides

Photographic procedure. Cells that had migrated into the filters were photographed at different levels within the filters by means of a Leitz Orthomat-W fully automatic photomicroscope, on which the distance between two divisions on the micrometer screw corresponds to appr. 1 µm in focal depth A high contrast lithographic film – Kodalith Ortho, Kodak Company – was found to be the most suitable The best results were achieved when the film was exposed as if it had an optical sensitivity of 6 Din (3 ASA). Correction of the light source became optimal by the inclusion of a green filter. The films were developed as prescribed by the manufacturer, projected on paper and counted with an automatically recording marker pencil.

Experimental design Blood leukocytes were isolated from five healthy staff members on five occasions In two experiments, six filters were prepared as described and incubated for 30 min. In another two experiments, eight filters were prepared and incubated for 180 min In one experiment, 14 filters were prepared, six of which were incubated for 30 min and the remaining eight for 180 min. Within each filter four randomly selected fields were photographed (magnification 25 × 3 2 × 10) at each of seven levels, beginning 25 µm from the cell-starting side and advancing in steps of 15 µm (Fig 1, 2 and 3). Cells on the cell-starting side and on the distal surface of the filter were counted photographically once on each filter, except for one experiment in which only one count was obtainable In the 180 min experiments, 'direct' differential counts of cells on the distal surface of the filter were made, once for each filter.

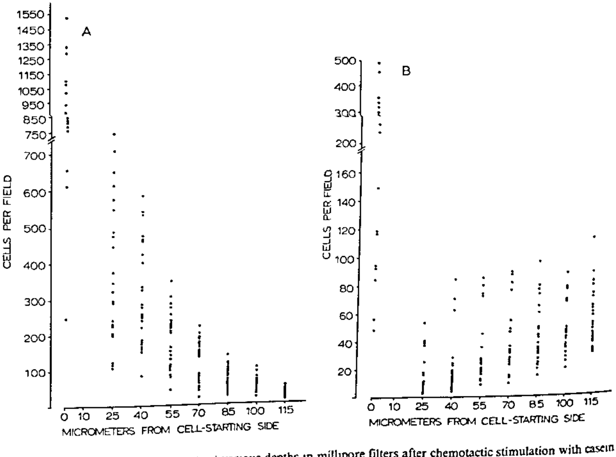


Fig 1 A and B Individual cell counts at various depths in millipore filters after chemotactic stimulation with casein A 30 min incubation, B 180 min incubation

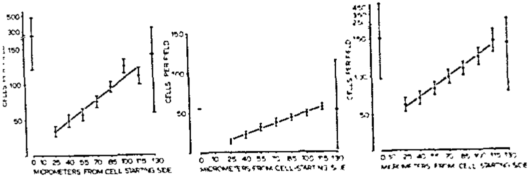


Fig 2 Mean leukocyte counts ± SEM at various depths in millipore filters after chemotactic stimulation with casein and 30 min incubation Counts from the cell-starting side are indicated at distance zero, and counts from the distal surface at distance 130 µm (mean & range) Data from three experiments

RESULTS

Figure 1 shows plots of individual cell counts against the corresponding distance from the cell-starting side in two randomly selected experiments, after incubation for 30 min (Fig 1 A) and after 180 min (Fig 1 B) Mean counts across all filters are plotted for each of three experiments from the 30 min group in Fig 2 and from the 180 min group in Fig 3 More than 95 per cent of cells that had reached the distal surface of filters were granulocytes

Statistics

In the 30 min experiments, the standard deviation (SD) for counts from different fields on the same level in each filter decreased monotonically from 218 at 25 µm to 2 7 at 115 µm which equalled 66 to 4 per cent of the corresponding level mean The same monotonic decrease was found for the standard error of the mean (SEM) across all filters for each level (Fig. 2) In the 180 min experiments, *SD ranged from 138 to 0 5 (108 to 2 per cent of the* level mean) with a tendency toward increasing spread with increasing distance from the cell-starting side (Fig. 3) In both groups, the data from individual filters in each experiment were more homogeneous than the data across all filters The mean cell-count distribution in each of the 30 min experiments (Fig. 2) was well described by a second degree polynomial (Residual Square Mean 10015, 4692 and 10669)[a])

There was no significant differences between the regression coefficients or intercepts ($p > 0\ 07$)[b]), but a close correlation was found between intercepts and corresponding regression coefficients ($R = -0\ 9879$)[c]). In the 180 min experiments, each mean cell-count distribution (Fig. 3) came close to a linear function ($p < 0\ 001$)[d]) The regression coefficients were all positive. There was no significant diffe-

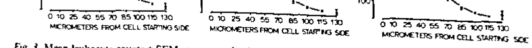


Fig 3 Mean leukocyte counts ± SEM at various depths in millipore filters after chemotactic stimulation with casein and 180 min incubation Counts from the cel-starting side are indicated at distance zero, and counts from the distal surface at distance 130 µm (mean & range) Data from three experiments.

Chemotaxis Modified Boyden chambers from Neuroprobe, Bethesda, Maryland, U.S A., and nitrocellulose filters from Millipore Filter Corp , Bedford, Massachusetts, U S A. (SSWPO 1300 lot 129281) with a pore size of 3 µm, were used in all experiments. Leukocytes were applied to the filters by centrifugation of 0.4 ml cell suspension in a Shandon Elliot cytocentrifuge at 500 × *g* for 3 min Each filter was rapidly transferred to a chamber, the lower compartment of which had been filled with a mixture of 80 per cent saturated casein in Hanks' solution (appr. 4 mg per ml) and 20 per cent pooled, heat activated (37° C for 15 min) human serum The upper compartment was filled with 2 per cent human serum albumin in Hanks' solution, and the chambers were incubated at 37° C at 100 per cent relative moisture. After the incubation period (*vide infra*), the filters were carefully transferred to a staining rack, fixed in a mixture of 10 per cent methanol in absolute ethanol, dehydrated in increasing concentrations of ethanol, stained with haematoxylin, made optically homogenous with xylene and mounted on glass slides.

Photographic procedure. Cells that had migrated into the filters were photographed at different levels within the filters by means of a Leitz Orthomat-W fully automatic photomicroscope, on which the distance between two divisions on the micrometer screw corresponds to appr. 1 µm in focal depth A high contra[illegible] lithographic film – Kodalith Ortho, Kodak Company was found to be the most suitable The best results we[illegible] achieved when the film was exposed as if it had [illegible] optical sensitivity of 6 Din (3 ASA) Correction of t[illegible] light source became optimal by the inclusion of a gre[illegible] filter. The films were developed as prescribed by t[illegible] manufacturer, projected on paper and counted with [illegible] automatically recording marker pencil

Experimental design. Blood leukocytes were isola[illegible] from five healthy staff members on five occasions. [illegible] two experiments, six filters were prepared as describ[illegible] and incubated for 30 min In another two experimen[illegible] eight filters were prepared and incubated for 180 min [illegible] one experiment, 14 filters were prepared, six of whi[illegible] were incubated for 30 min and the remaining eight f[illegible] 180 min. Within each filter four randomly selected fiel[illegible] were photographed (magnification 25 × 3 2 × 10) at ea[illegible] of seven levels, beginning 25 µm from the cell-starti[illegible] side and advancing in steps of 15 µm (Fig. 1, 2 and [illegible] Cells on the cell-starting side and on the distal surface [illegible] the filter were counted photographically once on ea[illegible] filter, except for one experiment in which only one cou[illegible] was obtainable In the 180 min experiments, dire[illegible] differential counts of cells on the distal surface of t[illegible] filter were made, once for each filter

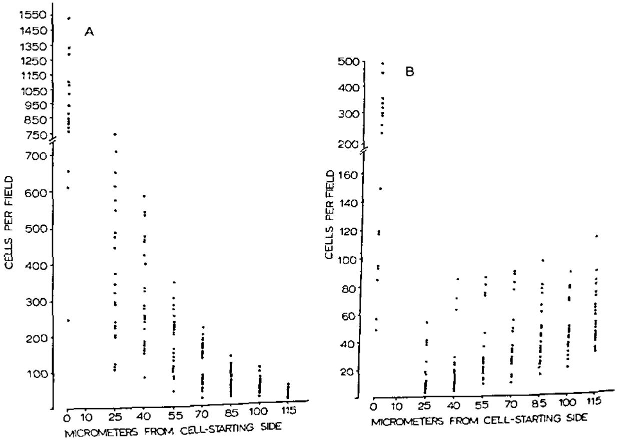


Fig 1 A and B Individual cell counts at various depths in millipore filters after chemotactic stimulation with casein A 30 min incubation, B 180 min incubation.

the study of the kinetics of whole cell populations, which appear to be an important basis of the counting methods in use at present.

The skilled technical assistance by Mrs. *Elin Askeland* is gratefully acknowledged. I am also indebted to *Rolv Skjærven* M.S. for statistical guidance and performance of statistical analyses.

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rence between intercepts or regression coefficients ($p > 0.18$)[b] and no correlation between intercepts and corresponding regression coefficients in this group ($R = 0.1953$)[c].

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[a] Biomedical computer programs BMDP5R. Health sciences computing facility. Department of Biomathematics, School of Medicine, Los Angeles, U.S.A.
[b] Kruskal-Wallis H-test
[c] Pearson correlation coefficient
[d] Student t-test

DISCUSSION

If cells which have migrated through the entire depth of Boyden filters accumulate on the distal surface, counting of these cells is actually a velocity measurement of the fastest moving fraction of the cell population. The same considerations can be made for double-filter techniques in which the migration is either stopped by a second small pore-size filter and the cells that have accumulated between the filters are counted, or the leading portion of cells is allowed to enter a second filter of equal pore size to be counted there, visually or by radio-assay. Finally, the leading front method is nothing but a velocity measurement of the fastest moving two (three) cells. A condition for the validity of such countings is the existence of a uniformity in the kinetics of whole cell populations when they are exposed to an excitant. *Zigmond & Hirsch* (1973) found such uniformity in random migration of horse granulocytes. They also found the distance travelled by the leading front to be a valid index for the behaviour of the total cell population. Recently, *Swanson & Becker* (1976) found a uniformity in migration of human neutrophils by the use of a chemotactic factor. However, by adding p-nitrophenyl-4-chlorobutyl phosphonate to the cell-side compartment, these investigators found the expected inhibition of migration by counting the total number of stimulated cells, whereas they found enhancement by use of the leading front method. The present results obtained with casein as attractant also showed a uniformity in the distribution of human granulocytes within millipore filters However, the exponential type of distribution after 30 min incubation (Fig 2) and the linear type after 180 min (Fig 3) appear to represent only fractions of the kinetic picture representative of the total cell population. In these experiments, in-filter counting at a distance of, for example, 50 µm from the cell-starting side of the filters, would have given approximately the same result after incubation for 30 and 180 min. A positional change of the plane in which counts are made, for example from 50 to 100 µm, is analogous to a small reduction in incubation time at an unchanged counting level. In the present experiments, such a change would have resulted in a considerable decrease in cell count after incubation for 30 min, whereas a similar change after 180 min would have caused a slight increase in the count Because increasing chemotactic activity accelerates migrating leukocytes (*Wilkinson* 1974), use of a weaker attractant in these experiments would have made the cell distribution curves appear further to the left in the diagrams. Therefore, a weaker attractant would have yielded lower counts after incubation for 30 min, whereas higher counts would have been the result after 180 min Although easier to count and less subjective than direct counting under the microscope, the photographically obtained counts showed only a moderate filter-to-filter reproducibility. In the 30 min experiments, the close correlation found between the regression curve intercepts, which represent the regressive cell-count at distance zero, and the corresponding regression coefficient, indicates that in these experiments the cell load is important for the distribution of cells within the filter Therefore, the considerable variations in cell load on the starting side of filters (Fig. 2) may be partially responsible for the variation in in-filter counts from filter to filter. The reason why no correlation existed between intercepts and corresponding regression coefficients in the 180 min experiments remains unclear. The finding that in each experiment the intercept of regression curves was below the corresponding cell-count on the starting side of the filter indicates that only a fraction of the cell population – evidently the granulocytes – participate in migration. The counts on the distal surface of filters in 30 min experiments (Fig. 2) were in accordance with extrapolated values from the regression curves. However, in 180 min experiments distal surface counts showed great variations (Fig. 3). Therefore, in the long incubation experiments, counting on the distal surface would have given no relevant information Comparison of intercepts and regression coefficients for regression curves in each group of experiments indicated no significant difference from person to person. The present results show the great importance of choosing the suitable incubation time when Boyden's technique is used in any modification They also show that the counts are dependent on the cell load, and that distal surface counting is useless when long periods of incubation are used (*e.g.* 3 hours). The reproducibility might be improved if the difference in cell load from filter to filter could be reduced. The in-filter method, modified by the photographic technique presented, is valuable for measuring chemotaxis. It may also prove useful for

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PRECIPITATING ANTIBODIES AGAINST *NEISSERIA MENINGITIDIS* IN NORMAL SERA AND THEIR POSSIBLE ORIGIN

GERD EVA HOFF and NIELS HØIBY

Statens Seruminstitut, Departments of Clinical Microbiology at Rigshospitalet and Hvidovre Hospital, Copenhagen, Denmark

Hoff, G. E. & Høiby, N. Precipitating antibodies against *Neisseria meningitidis* in normal sera and their possible origin. Acta path. microbiol. scand. Sect. C, 86: 123–129, 1978.

Crossed immunoelectrophoresis was used to study precipitating antibodies against *Neisseria meningitidis* in sera from 214 normal persons and in human gammaglobulin. A polyspecific meningococcal antigen preparation and a corresponding rabbit antiserum constituted the reference system. All the sera contained one to five precipitins against *N. meningitidis* which could be identified and quantified by means of the reference system. The precipitin score, which expresses the number and titre of precipitins in each serum, increased with age. More than 25 precipitins were found in the gammaglobulin preparation including antibodies against the groupspecific polysaccharides A, B and C. Absorption of three of the five normally occurring precipitins (nos. 4, 19, 22) with antigens from various bacteria showed that precipitins nos. 4 and 19 could be completely absorbed by antigens from other *Neisseria* species, but not by antigens from other genera. This indicates that these two precipitins may have been induced by non-pathogenic *Neisseria* species or by meningococci. The finding of protective antibodies in gammaglobulin might indicate the possibility of its use in prophylaxis or therapy against meningococcal infections.

Key words: *Neisseria meningitidis*; crossed immunoelectrophoresis; serum antibodies.

Gerd Eva Hoff, Department of Clinical Microbiology at Rigshospitalet, Afsnit 7806, Tagensvej 18, DK-2200 Copenhagen N, Denmark.

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A polyspecific antibody response to *Neisseria meningitidis* has been shown in serum from patients with meningococcal infection (11). However, patients with meningitis of other aetiology also had antibodies against meningococci (11). This raises the question as to whether even normal persons have antimeningococcal antibodies, either species-specific or cross-reactive, which have been induced by other bacteria, since *N. meningitidis* is known to share cross-reactive antigens with a large number of other bacterial species (12).

The aim of this study was to determine, by means of sensitive crossed immunoelectrophoretic methods, the occurence, specificities and titres of precipitating antibodies against *N. meningitidis* in serum from normal persons, and to evaluate whether such antibodies might be induced by other bacteria.

MATERIALS AND METHODS

Normal sera. Sera from 214 normal persons without known infectious or immunological disorders were examined. None of the persons had a known history of meningococcal infection. The material comprised 12 newborns (cord blood), 44 children examined in connection with parenthood questions, 29 children from a paediatric ward hospitalized for nocturnal enuresis, epilepsy, congenital heart disorders, adiposity or social reasons, 32 persons from the laboratory and hospital staff and their relatives, 65 blood donors, and 32 persons

TABLE 1 *Number and Percentage of Normal Persons with Neisseria meningitidis Precipitins Nos 19, 22 and 55 Distributed According to Titre Class*

Precipitins	No precipitins Number	(%)	Titre 1–3 Number	(%)	Titre 4–6 Number	(%)	Titre 7–9 Number	(%)
No 19	0		153	(72)	56	(26)	5	(2)
No 22	7	(3)	168	(79)	39	(18)	0	
No 55	30	(14)	101	(47)	83	(39)	0	

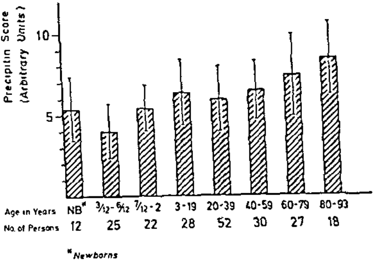


Fig 2 Mean precipitin score within the given age groups ± 1 SD The precipitin score expresses both number of precipitins and their titres

The number of precipitins per serum ranged from 1 to 5 (mean 3 2) In Table 1, which shows the titres of the three most prevalent precipitins, it can be seen that precipitins against antigen no 19 are present in 100%, against no 22 in 97% and against no 55 in 86% of the sera Precipitins against antigens nos 4 and 48 were found in 5% and 15% of the sera, respectively A few other sera (1 2% showed additional precipitins which, however, could not be identified with certainty in the reference pattern

A precipitin score was calculated for each serum, and the distribution according to age is shown in Fig 2 The lowest score was found at the age of 3–6 months, after which it increased with age to twice as high values in the oldest persons As regards the number of precipitins, the age variation was less obvious (mean 3 2 precipitins per person, range 2 6–3 6)

Gammaglobulin

The pooled plasma from which the gammaglobulin was prepared showed precipitin scores similar to those of normal serum However, as shown in Fig 3, when the concentrated gammaglobulin was incorporated in the intermediate gel in the reference system, at least 25 precipitins were found, including nos 2 and 7 directed against the group-specific polysaccharides A and C, respectively. Antibodies against the group-specific polysaccharide B could also be demonstrated in gammaglobulin when group B antiserum was used in the reference gel (Fig. 4). Only minor quantitative differences between the nine gammaglobulin batches were found.

Absorption Experiments

When the meningococcal Ref-ag was run against gammaglobulin incorporated in the reference gel (a

from an old peoples' home. Some of the sera had been used in a previous study (14).

Gammaglobulin Nine batches of 10% human gammaglobulin produced according to *Deutsch et al.* (4), as well as their corresponding plasma pools, were studied. The gammaglobulin batches are included in the commercial gammaglobulin from Statens Seruminstitut, Copenhagen. Each batch originates from serum from at least 2000 normal subjects.

Immunoelectrophoretic Methods

Crossed immunoelectrophoresis with intermediate gel was used to detect human antibodies against meningococci and was performed as described previously (11). Briefly, 2 µl *N meningitidis* antigen (Ref-ag) was run against 75 µl of the corresponding rabbit antiserum (Ref-ab) in 1.875 ml gel (5 µl/cm²) on 5 × 5 cm glass plates The Ref-ab contains 63 precipitins against Ref-ag, including those against the group-specific polysaccharides A and C, but not including precipitins against the group-specific polysaccharide B (11). An intermediate gel with 200 µl saline, normal serum or plasma pools in 0 8 ml gel (33.3 µl/cm²) was interposed between first and second dimension runs.

5 × 7 cm glass plates were used for the gammaglobulin 1 ml gammaglobulin was incorporated in 1 15 ml intermediate gel (62 5 µl/cm²). The reference gel was composed of either 100 µl Ref-ab in 0.9 ml gel (10 µl/cm²) or 400 µl meningococcal group B antiserum (11) in 0 6 ml gel (40 µl/cm²). Since the high concentration of gammaglobulin in the intermediate gel caused a certain amount of unspecific background staining, and as the electro-endosmotic properties of the gel tended to make the reference pattern move into the intermediate gel, an »empty« gel, with saline was always interposed between the reference gel and the intermediate gel

The human precipitins were identified in relation to the reference system (2) Quantification of antibodies in the human sera was carried out by comparison of the area included by each precipitate in the intermediate gel containing human serum with the corresponding areas in eleven standard plates with dilutions of Ref-ab in the intermidiate gel (11). The titres of the human precipitins were then expressed in relation to the concentrations of Ref-ab in the standard plates (titre 1–12) (20) The precipitin score for each serum was calculated according to *Axelsen et al* (3), as described previously (11). The precipitin score includes both the number of precipitins and the titres of each of the precipitins

Crossed-line immunoelectrophoresis was used for absorption of antibodies *in situ* experiments (18). 400 µl gammaglobulin was used in the 1.5 ml reference gel (26 7 µl/cm²) The antigen concentration in the intermediate gel was 33 3 µl/cm² Antigens from the following bacteria were included (see Table 2). *Pseudomonas aeruginosa* (16), *Bordetella pertussis* (10), *Escherichia coli*, *Klebsiella pneumoniae* type 35, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* group A, *Branhamella catarrhalis*, *Neisseria subflava*, *Neisseria sicca* and *Neisseria perflava*. The preparation of the antigens and technical details concerning the electrophoresis have been described elsewhere (12, 13, 17).

The degree of absorption of *N. meningitidis* antibodies was estimated by comparison of the area included by a precipitate after absorption with areas of this precipitate on four standard plates with decreasing amounts of gammaglobulin in the reference gel (100%, 75%, 50% and 25%) In this way, the degree of cross-reaction can be expressed as 0%, 25–50%, 50–75%, 75–100% and 100%, respectively. Absorption of less than 25% is considered to be insignificant, due to the analytical variation of the present method (15)

RESULTS

Normal Sera

All normal sera contained precipitins against *N meningitidis*, an example of which is given in Fig 1.

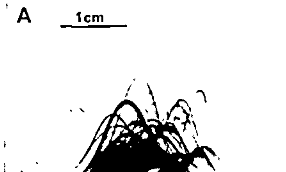


Fig 1. Crossed immunoelectrophoresis of *Neisseria meningitidis* antigens (Ref-ag) against a corresponding antiserum (Ref-ab) with intermediate gel containing saline (A) or normal serum (B) The three precipitates usually found in normal serum are indicated by arrows and numbers. (Technical. anode to the right in the first dimension and at the top in the second dimension electrophoresis. Staining· Coomassie brilliant blue)

TABLE I *Number and Percentage of Normal Persons with Neisseria meningitidis Precipitins Nos 19, 22 and 55 Distributed According to Titre Class*

Precipitins	No precipitins Number	(%)	Titre 1–3 Number	(%)	Titre 4–6 Number	(%)	Titre 7–9 Number	(%)
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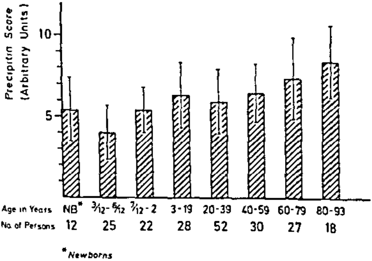


Fig 2 Mean precipitin score within the given age groups ± 1 SD The precipitin score expresses both number of precipitins and their titres

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A precipitin score was calculated for each serum, and the distribution according to age is shown in Fig 2 The lowest score was found at the age of 3–6 months, after which it increased with age to twice as high values in the oldest persons As regards the number of precipitins, the age variation was less obvious (mean 3.2 precipitins per person, range 2 6–3 6)

Gammaglobulin

The pooled plasma from which the gammaglobulin was prepared showed precipitin scores similar to those of normal serum However, as shown in Fig 3, when the concentrated gammaglobulin was incorporated in the intermediate gel in the reference system, at least 25 precipitins were found, including nos. 2 and 7 directed against the group-specific polysaccharides A and C, respectively. Antibodies against the group-specific polysaccharide B could also be demonstrated in gammaglobulin when group B antiserum was used in the reference gel (Fig 4) Only minor quantitative differences between the nine gammaglobulin batches were found.

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When the meningococcal Ref-ag was run against gammaglobulin incorporated in the reference gel (a

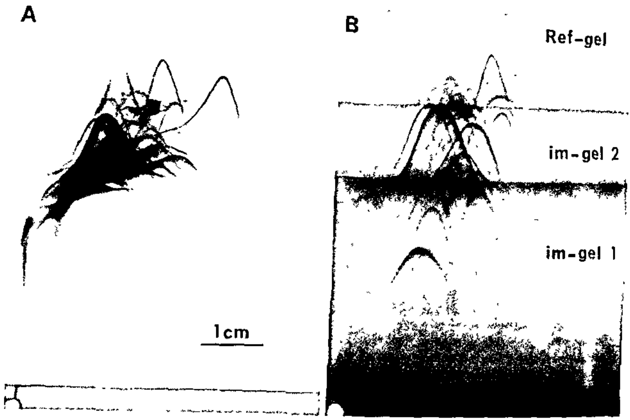


Fig 3. Crossed immunoelectrophoresis of *Neisseria meningitidis* antigens (Ref-ag) against a corresponding antiserum (Ref-ab) in the reference gel (Ref-gel) The second intermediate gel (Im-gel 2) contains saline. A: saline in the first intermediate gel (Im-gel 1); the meningococcal reference pattern can be seen. B. 10% concentrated gammaglobulin in the first intermediate gel. At least 25 precipitates have been pulled down, thus indicating the presence of antibodies against the corresponding antigens in the gammaglobulin preparation. (Technical: see Fig. 1.)

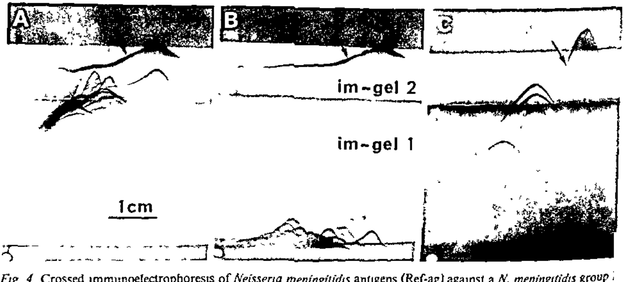


Fig 4 Crossed immunoelectrophoresis of *Neisseria meningitidis* antigens (Ref-ag) against a *N. meningitidis group* B antiserum in the reference gel (Ref-gel) The second intermediate gel (Im-gel 2) contains saline A. saline in the first intermediate gel (Im-gel 1). The precipitate corresponding to the meningococcal group B polysaccharide is indicated by an arrow *It can be seen that the group B antiserum also contains other meningococcal precipitins. B. N. meningitidis* antiserum (Ref-ab) in the first intermediate gel The Ref-ab contains precipitins against the antigens in the Ref-ag except the group B polysaccharide (11), and thus all precipitates are pulled down except that corresponding to group B polysaccharide. C. 10% human gammaglobulin in the first intermediate gel. The precipitate corresponding to group B *polysaccharide has been pulled down, thus indicating the presence of antibodies against this antigen* in the gammaglobulin preparation (Technical. *see Fig. 1.)*

TABLE 2 *Absorption of the Three* Neisseria meningitidis *Precipitins (Nos 4, 19, 22) Regularly Demonstrable in Human Gammaglobulin with Antigens from Other Bacteria*

	Cross-reactive antibodies and degree of cross-reactivity 100%	100–≧75%	75–≧50%	50–≧25%
Pseudomonas aeruginosa (4, O groups 3, 5, 6, 11)	22		19	
Escherichia coli (2, rough and O21 H27)	22		19	
Bordetella pertussis (4, St. 3803–3825–3843–3860)	22	19		
Klebsiella pneumoniae (1, type 35)			19,22	
Staphylococcus aureus (4, from each of four phage groups)	22			
Streptococcus pneumoniae (1)				22
Streptococcus pyogenes (1, group A)				22
Branhamella catarrhalis (2, ATCC 8193 and NCTC 4103)	22		19	
Neisseria subflava	4,19,22			
N sicca (1, ATCC 9913)	4,19,22			
N perflava (1, ATCC 10555)	4,19,22			

less sensitive technique), only three precipitates were visible regularly, corresponding to antigens nos. 4, 19 and 22, whereas four other precipitates were very faint and only visible occasionally (nos. 2, 7, 48 and 55)

Antibodies against antigen no. 22 could be completely absorbed by antigens from all bacteria examined, except the genus *Streptococcus* Antibodies against no. 19 could be absorbed by all gram-negative bacteria examined, but 100% absorption could only be effected by antigens from other *Neisseria* species, the only species which could also absorb antibodies against antigen no 4 (Table 2)

DISCUSSION

The study of serum shows that practically all normal persons examined had antibodies against meningococcal antigens nos. 19, 22 and 55, and a few persons also against antigens nos. 4 and 48. However, this seems to be only the »top of the iceberg« (1), because when concentrated gammaglobulin was examined, at least 25 precipitins were revealed, most of them low-titred and only detectable by means of the sensitive intermediate gel technique The possibility that the high number of precipitins in gammaglobulin might originate from a few hyperimmune sera is less likely, since such sera have not been found among the normal sera examined, or among meningococcal convalescent sera (11).

In a previous study on the occurrence of antibodies against *P. aeruginosa* in normal sera, precipitins against one antigen (corresponding to meningococcal antigen no. 19 (12)) were found in only half of the sera and against one other antigen in 4% of the sera (14) The much higher prevalence of sera with antibodies against some meningococcal antigens (average 3.2) may be because meningococcal antigens show 100% cross-reactivity with most antigens from other *Neisseria* species (12) and because these species are members of the normal flora. It is possible, therefore, that antibodies against the cross-reactive antigens do not reflect prior immunization with meningococci, but rather with

the cross-reactive *Neisseria* species. This applies e.g. to antibodies against antigens nos. 4 and 55 which cross-react with antigens from other *Neisseria* species but not with antigens from 21 other bacterial species (12). The three other antigens, nos. 19, 22 and 48, against which antibodies occur in normal sera, have been shown previously to cross-react with many other bacterial genera also (12).

Only 75% of antibodies in gammaglobulin against meningococcal antigen no. 19 could be absorbed by other gram-negative genera, *e.g. P. aeruginosa*, whereas 100% of normally occurring antibodies against the same *P. aeruginosa* antigen (no. 10 (12)) could be absorbed by several other gram-negative bacteria including *N. meningitidis*. This indicates that although 75% of the normally occurring antibodies against antigen no. 19 might be induced by several other gram-negative bacteria, the remaining 25% of these antibodies must be induced by bacteria belonging to the *Neisseria* species. These results are in accordance with the previous finding (14) that antibodies against *P. aeruginosa* antigen no. 10 were present in only 54% of normal sera.

The degree of cross-reaction was 25–50% between meningococcal antigen no. 22 and the other gram-negative bacteria employed in this study (12). This means that only 25–50% of the rabbit antibodies raised against antigen no. 22 could be absorbed by antigens from these other bacteria. Human antibodies against antigen no. 22 in gammaglobulin could be absorbed completely by other bacterial genera. The antibodies in gammaglobulin against meningococcal antigen no. 22 are thus directed against a common antigenic determinant of this molecule and may be induced by some bacteria other than *Neisseria*.

The origin of antibodies against antigen no. 48, which was shown previously to cross-react with a few genera of gram-negative bacteria (12), could not be elucidated in this study

Antibodies against the group-specific polysaccharides A, B and C were also detected in human gammaglobulin preparations. The antibodies against polysaccharides A and C were high-titred, in contrast to the low-titred antibodies against group B polysaccharide. The fact that such antibodies against the group-specific B polysaccharide could be found in gammaglobulin was somewhat surprising, since this polysaccharide is known to be a poor immunogen (8, 23). It is notable, however, that antibodies against group B polysaccharide were also found in five out of seven patients infected with group B meningococci (11) using the same group B antiserum in the reference gel as in this study.

Antibodies against the meningococcal polysaccharides may also be induced by other bacteria, since some widely occurring bacteria have been found to possess capsules which cross-react with meningococcal polysaccharides A, B and C. Strains of *Bacillus pumilis* have been shown to produce a polysaccharide which is antigenically and structurally related to *N. meningitidis* group A polysaccharide (19, 21), and the K1 and K92 antigens of *E. coli* have been shown to be antigenically and structurally related to *N. meningitidis* group B and C polysaccharides, respectively (6, 9).

Antibodies against the group-specific polysaccharides A and C are known to confer protection against infection with the corresponding meningococcal groups (7, 22), and small amounts of antibodies against group B polysaccharide are known to add considerably to the immuno-defense against group B disease in the presence of type-specific antibodies (5). The clinical implication of this study may therefore be that gammaglobulin may prove valuable as prophylaxis or in the therapy of meningococcal infections.

Some of the sera were kindly provided by Dr. *Peter Bækgaard*, Department of Obstetrics YA, Rigshospitalet, and Dr. *Klavs Henningsen*, Department of Serology, Institute of Forensic Medicine, University of Copenhagen. The gammaglobulin preparations and plasma pools were obtained by courtesy of Dr. *Poul Hansen*, Department of Blood Fractionation, Statens Seruminstitut, Copenhagen, and the meningococcal group B antiserum by courtesy of Dr. *J. B. Robbins*, Bureau of Biologics, FDA, Bethesda, Maryland, U.S.A.

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the cross-reactive *Neisseria* species. This applies e.g. to antibodies against antigens nos. 4 and 55 which cross-react with antigens from other *Neisseria* species but not with antigens from 21 other bacterial species (12). The three other antigens, nos. 19, 22 and 48, against which antibodies occur in normal sera, have been shown previously to cross-react with many other bacterial genera also (12).

Only 75% of antibodies in gammaglobulin against meningococcal antigen no. 19 could be absorbed by other gram-negative genera, *e.g. P. aeruginosa*, whereas 100% of normally occurring antibodies against the same *P. aeruginosa* antigen (no. 10 (12)) could be absorbed by several other gram-negative bacteria including *N. meningitidis*. This indicates that although 75% of the normally occurring antibodies against antigen no. 19 might be induced by several other gram-negative bacteria, the remaining 25% of these antibodies must be induced by bacteria belonging to the *Neisseria* species. These results are in accordance with the previous finding (14) that antibodies against *P. aeruginosa* antigen no. 10 were present in only 54% of normal sera.

The degree of cross-reaction was 25–50% between meningococcal antigen no. 22 and the other gram-negative bacteria employed in this study (12). This means that only 25–50% of the rabbit antibodies raised against antigen no. 22 could be absorbed by antigens from these other bacteria. Human antibodies against antigen no. 22 in gammaglobulin could be absorbed completely by other bacterial genera. The antibodies in gammaglobulin against meningococcal antigen no. 22 are thus directed against a common antigenic determinant of this molecule and may be induced by some bacteria other than *Neisseria*.

The origin of antibodies against antigen no. 48, which was shown previously to cross-react with a few genera of gram-negative bacteria (12), could not be elucidated in this study.

Antibodies against the group-specific polysaccharides A, B and C were also detected in human gammaglobulin preparations. The antibodies against polysaccharides A and C were high-titred, in contrast to the low-titred antibodies against group B polysaccharide. The fact that such antibodies against the group-specific B polysaccharide could be found in gammaglobulin was somewhat surprising, since this polysaccharide is known to be a poor immunogen (8, 23). It is notable, however, that antibodies against group B polysaccharide were also found in five out of seven patients infected with group B meningococci (11) using the same group B antiserum in the reference gel as in this study.

Antibodies against the meningococcal polysaccharides may also be induced by other bacteria, since some widely occurring bacteria have been found to possess capsules which cross-react with meningococcal polysaccharides A, B and C. Strains of *Bacillus pumilis* have been shown to produce a polysaccharide which is antigenically and structurally related to *N. meningitidis* group A polysaccharide (19, 21), and the K1 and K92 antigens of *E. coli* have been shown to be antigenically and structurally related to *N. meningitidis* group B and C polysaccharides, respectively (6, 9).

Antibodies against the group-specific polysaccharides A and C are known to confer protection against infection with the corresponding meningococcal groups (7, 22), and small amounts of antibodies against group B polysaccharide are known to add considerably to the immuno-defense against group B disease in the presence of type-specific antibodies (5). The clinical implication of this study may therefore be that gammaglobulin may prove valuable as prophylaxis or in the therapy of meningococcal infections.

Some of the sera were kindly provided by Dr. *Peter Bækgaard*, Department of Obstetrics YA, Rigshospitalet, and Dr. *Klavs Henningsen*, Department of Serology, Institute of Forensic Medicine, University of Copenhagen. The gammaglobulin preparations and plasma pools were obtained by courtesy of Dr. *Poul Hansen*, Department of Blood Fractionation, Statens Seruminstitut, Copenhagen, and the meningococcal group B antiserum by courtesy of Dr. *J. B. Robbins*, Bureau of Biologics, FDA, Bethesda, Maryland, U.S.A.

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INHIBITORY AND STIMULATORY EFFECT OF SPLEEN CELLS FROM TUMOUR BEARING ANIMALS ON THE GROWTH OF SYNGENEIC TUMOUR CELLS

F S LARSEN and J V. SPÄRCK

The Immunological Laboratory, Statens Seruminstitut, Copenhagen, Denmark

Larsen, F S & Sparck, J V Inhibitory and stimulatory effect of spleen cells from tumour bearing animals on the growth of syngeneic tumour cells Acta path microbiol scand Sect C, 86 131-139, 1978

When spleen cells from C3H mice inoculated with a primary spontaneously arisen C3H mammary carcinoma were added to tumour target cells of the same type *in vitro*, both an inhibitory and a stimulatory effect on target cell growth were seen, when compared with the effect of adding normal syngeneic spleen cells The inhibitory effect was seen regularly when high concentrations of spleen cells were added, while there was a stimulatory effect when low concentrations were added to the tumour target cells When transferring spleen cells from tumour bearing mice together with a tumour inoculum to groups of normal syngeneic recipients, the resultant tumour growth was enhanced, as compared with recipients given tumour plus normal spleen cells and those given tumour cells only It was found that the spleen cells which caused the greatest enhancement *in vivo* caused the greatest inhibition *in vitro* This relationship could be explained by assuming the occurrence in spleens of tumour bearers of a population of reactive cells which when added in high concentrations to tumour target cells *in vitro* cause inhibition, while they cause stimulation of tumour growth *in vivo* because of being transferred to recipients in relatively low concentrations

Key words Spleen cells, tumour bearing mice, syngeneic tumour cell growth

Flemming S Larsen, Immunological Laboratory, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark

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The finding that lymphocytes from tumour bearers may be cytotoxic against tumour cells of the same type *in vitro* gave support to the assumption that these cells constitute an immune defence against the tumour. The apparent paradox that tumours grow in spite of the presence of immune cells could be explained by the existence of blocking serum factors, as suggested by the finding that serum of tumour bearers often contain substances inhibitory to the *in vitro* cytotoxic reaction between lymphocytes and the tumour (7)

However, when testing this *in vitro* reaction between lymphocytes and tumour target cells, sometimes also a target cell growth stimulatory activity of lymphocytes from tumour bearing animals was seen, as compared with lymphocytes from normal animals (2, 11) This stimulatory activity was found when »low« lymphocyte tumour cell ratios were tested (500 1), while target cell inhibition often was found with higher ratios (4, 10)

Furthermore, it was found that lymphoid cells from tumour bearing animals enhance tumour growth *in vivo* after transfer together with a tumour inoculum to syngeneic recipients, as compared with transfer of normal lymphocytes (5, 12, 13, 17, 18). Such an enhancement was found when the spleen cells were taken from donor mice at a late stage of tumour growth, while spleen cells from an early stage caused inhibition of tumour growth (5, 18). It has also been shown that the inhibitory and stimulatory activities of spleen cells were associated with separable cell populations (13), indicating the

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animals were distributed symmetrically on to the plate. One plate was used for each concentration of spleen cells to be tested, so that 12 wells received normal cells and 12 similarly situated wells received cells from the tumour bearing animals. Eight wells received medium only and served as medium controls. Usually several plates with different spleen cell concentrations were prepared. Incubation was performed for 3 days in sealed boxes containing humidified 5% CO_2 atmosphere. At the end of this period, the plates were washed by immersion in physiological saline until the non-adherent cells were removed. The plates were then fixed in methanol and stained with Giemsa stain.

Calculations. Percentage reduction was calculated from the formula

$$\frac{N_c - N_e}{N_c} \times 100\,\%$$

and percentage stimulation from

$$\frac{N_c - N_e}{N_e} \times 100\,\%$$

giving negative values. N_c and N_e are the average number of remaining target cells in normal cell control and in experimental wells, respectively.

RESULTS

Inhibition of Tumour Growth in vitro after Addition of Spleen Cells from Mice Inoculated with Tumour.

The MC-test is used widely as a method for detecting the reactivity of lymphoid cells from animal or man against tumours or allogeneic grafts. When using this test in different series of syngeneic tumour transplantations of spontaneously arisen C3H mamma carcinomas, transplantation of only one out of three tumours resulted in detectable and significant tumour cell inhibition or killing by the addition of spleen cells from the recipient mice, as compared with spleen cells from normal mice. This negative result when using some tumours was not due simply to inferior technique because, at the same time, a definite cytotoxic activity against the tumour cells of spleen cells from allogeneic immunized mice was seen.

In an attempt to increase the sensitivity of the MC-test, the spleen cells were incubated for 3 days with Con-A before addition to target cells. It was expected that, if not already present, cytotoxic

TABLE 1 *Addition to Tumour Target Cells of Preincubated or not Preincubated Spleen Cells from Mice Inoculated with Tumour*

Days after transplantation	Spleen cells added[a]	Remaining number and percentage reduction of tumour cells after addition of 10^5 spleen cells per well					
		No preincubation		Preincubation without Con-A		Preincubation with Con-A	
8	N	146	10% (ns)	17	12% (ns)	349	47% (sss)
	T	132		15		184	
15	N	185	31% (s)	27	79% (ss)	56	79% (sss)
	T	128		4		12	
22	N	81	−1% (ns)	62	89% (sss)	ND	
	T	82		7			
29	N	282	5% (ns)	49	94% (sss)	30	47% (s)
	T	268		3		16	

[a] N = spleen cells from normal animals
T = spleen cells from animals inoculated with tumour
ns = not significant.
s = significant, $p < 0.05$ (Students t-test)
ss = significant, $p < 0.01$.
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presence in the spleen of both inhibitory and stimulatory lymphoid cells.

It is still an open question whether the enhancing cells act as direct stimulatory cells or as cells suppressing immune resistance to the tumour. The former possibility was favoured by Sparck (14, 15) who, by transplanting mouse mammary adenocarcinomas, demonstrated that tumours grew less well in immunosuppressed syngeneic recipients, in contrast to what was found in the allogeneic combination. He formulated a theory of immune stimulation of tumours claiming that tumour growth is dependent on an active immune response of the host, and that a weak immune response promotes tumour growth, while a strong immune response inhibits the tumour.

The purpose of the present work was to study the *in vitro* reaction between tumour target cells and lymphoid cells from tumour bearing mice at different times after tumour inoculation and to study the effect of these lymphoid cells on tumour growth *in vivo* by transferring them together with the tumour inoculum to normal recipients.

MATERIALS AND METHODS

Tumours. The tumours used in this study were primary spontaneous C3H mamma carcinomas from a C3H line of low tumour frequency (< 15%) After having reached a suitable size, the tumours were removed, cut into small pieces and washed twice with prewarmed saline 10 ml of warm trypsin 0.2% in phosphate buffered saline was added and the suspension shaken for 5 minutes in a 37° C waterbath Then the supernatant was decanted through a gauze filter into 5 ml foetal calf serum and kept in the cold. The process was repeated until the tissue was exhausted. If the solution became viscous, 10–20 drops of a 0 04% DNase solution (Sigma Chemical Co. St Louis, Mo) were added. The pooled supernatants were centrifuged for 10 minutes at 300 × *g* and the cells suspended in RPMI-1640 (Gibco Bio Cult, Scotland) containing 20% foetal calf serum, 0 05 mg/ml streptomycin, 0 01 mg/ml neomycin and 1500 IU/ml penicillin and supplemented with 1% of a freshly thawed solution of 200 mM glutamin This medium will be referred to as 20%-RPMI The cell suspension was counted with trypan blue, and after centrifugation for 5 minutes at 400 × *g* was adjusted to 10^7 dye excluding cells per ml in a mixture of one part foetal calf serum and one part 20% dimethyl sulphoxide in phosphate buffer The suspension was sucked into straws containing 0 6 ml each and stored in liquid nitrogen after programmed freezing

Tumour transplantation. On the day of transplantation, a number of straws containing the frozen tumour cells were thawed by rapid transfer from the nitrogen container to tubes of 20%-RPMI prewarmed to 37° C. The straws were immediately cut open and removed, leaving the cells free. The suspensions were centrifuged for 5 minutes at 400 × *g*, the cells were resuspended in TC-199 (Difco) and washed once with this medium Viable cells were counted and the suspensions adjusted to the desired concentrations. The recipients were each given 0 1 ml subcutaneously. The tumours were fully transplantable and became palpable 3–4 weeks after inoculation The tumour growth was followed twice a week by measuring the two main diameters of each tumour with calipers. Regression was never seen

Tissue culture (target cells) Tumour cells were used as targets in the microcytotoxicity test (MC-test) of *Takasugi & Klein* (16). One straw of tumour cells was thawed as described above, and the cells were counted and resuspended in 20%-RPMI. 10 µl was dispensed into the wells of Falcon 3034 microtitre plates (Falcon Plastics, Los Angeles, Calif.) and incubated at 37° C in a sealed box containing 5% CO_2 atmosphere. The plating efficiency varied from tumour to tumour, but generally 500–1000 living tumour cells had to be seeded out in each well to get approximately 50 adherent cells on the next day.

Preparation of spleen cells for transfer. The mice were killed by cervical dislocation. The spleens were removed and forced gently through fine wire mesh into 20%-RPMI. The resulting suspension was homogenized carefully with a Pasteur pipette and filtered through a double layer of gauze. The cells were washed three times in 20%-RPMI by centrifuging for 5 minutes at 400 × *g* and adjusted to 5 × 10^7 viable cells per ml in TC-199 Just before transfer to the recipients, the spleen cells were mixed with tumour cells and an amount of 0 2 ml was injected subcutaneously.

Preparation of spleen cells for the MC-test Spleens were forced gently through fine wire mesh into about 4 ml 20%-RPMI. The suspension was homogenized carefully with a Pasteur pipette, layered on 3 ml »lymfoprep« (Nyegård & Co, Oslo) and centrifuged for 15 minutes at 400 × *g* The cells at the interphase (recovery 25%) were washed three times in 20%-RPMI by centrifuging for 5 minutes at 400 × *g* and were adjusted to 10^7 living cells per ml in 20%-RPMI, after which they were ready for the MC-test

Preincubation was performed in test tubes (Falcon 2001, Los Angeles, Calif) containing 5 × 10^6 cells in 2 ml 20%-RPMI Half of the tubes were usually given 20 µg/ml Con-A (Sigma Chemical Co. St Louis, Mo) The tubes were incubated for 2 or 3 days in 5% CO_2 atmosphere. At the end of this period the content of the tubes was pooled, centrifuged and resuspended in 20%-RPMI and adjusted to 10^7 viable cells per ml The number of viable cells at the end of the culture process was generally 40–50% of the initial number

Microcytotoxicity test One day after plating of the target cells the non-adherent cells were removed by inverting the plates and changing the medium The spleen cells were added immediately after the medium was again sucked away. Since the growth of target cells on the plate was reduced in the outermost wells, these received medium only In order to secure equivalent conditions for the cells to be tested, the normal spleen cells and the spleen cells from the tumour bearing

animals were distributed symmetrically on to the plate. One plate was used for each concentration of spleen cells to be tested, so that 12 wells received normal cells and 12 similarly situated wells received cells from the tumour bearing animals. Eight wells received medium only and served as medium controls. Usually several plates with different spleen cell concentrations were prepared. Incubation was performed for 3 days in sealed boxes containing humidified 5% CO_2 atmosphere. At the end of this period, the plates were washed by immersion in physiological saline until the non-adherent cells were removed. The plates were then fixed in methanol and stained with Giemsa stain.

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The purpose of the present work was to study the *in vitro* reaction between tumour target cells and lymphoid cells from tumour bearing mice at different times after tumour inoculation and to study the effect of these lymphoid cells on tumour growth *in vivo* by transferring them together with the tumour inoculum to normal recipients.

MATERIALS AND METHODS

Tumours The tumours used in this study were primary spontaneous C3H mamma carcinomas from a C3H line of low tumour frequency (< 15%). After having reached a suitable size, the tumours were removed, cut into small pieces and washed twice with prewarmed saline. 10 ml of warm trypsin 0.2% in phosphate buffered saline was added and the suspension shaken for 5 minutes in a 37° C waterbath. Then the supernatant was decanted through a gauze filter into 5 ml foetal calf serum and kept in the cold The process was repeated until the tissue was exhausted If the solution became viscous, 10–20 drops of a 0.04% DNase solution (Sigma Chemical Co., St. Louis, Mo.) were added The pooled supernatants were centrifuged for 10 minutes at 300 × *g* and the cells suspended in RPMI-1640 (Gibco Bio Cult, Scotland) containing 20% foetal calf serum, 0 05 mg/ml streptomycin, 0.01 mg/ml neomycin and 1500 IU/ml penicillin and supplemented with 1% of a freshly thawed solution of 200 mM glutamin This medium will be referred to as 20%-RPMI The cell suspension was counted with trypan blue, and after centrifugation for 5 minutes at 400 × *g* was adjusted to 10^7 dye excluding cells per ml in a mixture of one part foetal calf serum and one part 20% dimethyl sulphoxide in phosphate buffer The suspension was sucked into straws containing 0.6 ml each and stored in liquid nitrogen after programmed freezing.

Tumour transplantation. On the day of transplantation, a number of straws containing the frozen tumour cells were thawed by rapid transfer from the nitrogen container to tubes of 20%-RPMI prewarmed to 37° C. The straws were immediately cut open and removed, leaving the cells free. The suspensions were centrifuged for 5 minutes at 400 × *g*, the cells were resuspended in TC-199 (Difco) and washed once with this medium Viable cells were counted and the suspensions adjusted to the desired concentrations. The recipients were each given 0.1 ml subcutaneously. The tumours were fully transplantable and became palpable 3–4 weeks after inoculation The tumour growth was followed twice a week by measuring the two main diameters of each tumour with calipers Regression was never seen

Tissue culture (target cells) Tumour cells were used as targets in the microcytotoxicity test (MC-test) of *Takasugi & Klein* (16). One straw of tumour cells was thawed as described above, and the cells were counted and resuspended in 20%-RPMI. 10 µl was dispensed into the wells of Falcon 3034 microtitre plates (Falcon Plastics, Los Angeles, Calif.) and incubated at 37° C in a sealed box containing 5% CO_2 atmosphere The plating efficiency varied from tumour to tumour, but generally 500–1000 living tumour cells had to be seeded out in each well to get approximately 50 adherent cells on the next day.

Preparation of spleen cells for transfer. The mice were killed by cervical dislocation The spleens were removed and forced gently through fine wire mesh into 20%-RPMI. The resulting suspension was homogenized carefully with a Pasteur pipette and filtered through a double layer of gauze. The cells were washed three times in 20%-RPMI by centrifuging for 5 minutes at 400 × *g* and adjusted to 5 × 10^7 viable cells per ml in TC-199 Just before transfer to the recipients, the spleen cells were mixed with tumour cells and an amount of 0 2 ml was injected subcutaneously

Preparation of spleen cells for the MC-test Spleens were forced gently through fine wire mesh into about 4 ml 20%-RPMI The suspension was homogenized carefully with a Pasteur pipette, layered on 3 ml »lymfoprep« (Nyegård & Co , Oslo) and centrifuged for 15 minutes at 400 × *g* The cells at the interphase (recovery 25%) were washed three times in 20%-RPMI by centrifuging for 5 minutes at 400 × *g* and were adjusted to 10^7 living cells per ml in 20%-RPMI, after which they were ready for the MC-test

Preincubation was performed in test tubes (Falcon 2001, Los Angeles, Calif) containing 5 × 10^6 cells in 2 ml 20%-RPMI Half of the tubes were usually given 20 µg/ml Con-A (Sigma Chemical Co , St Louis, Mo) The tubes were incubated for 2 or 3 days in 5% CO_2 atmosphere At the end of this period the content of the tubes was pooled, centrifuged and resuspended in 20%-RPMI and adjusted to 10^7 viable cells per ml The number of viable cells at the end of the culture process was generally 40–50% of the initial number.

Microcytotoxicity test One day after plating of the target cells the non-adherent cells were removed by inverting the plates and changing the medium The spleen cells were added immediately after the medium was again sucked away Since the growth of target cells on the plate was reduced in the outermost wells, these received medium only. In order to secure equivalent conditions for the cells to be tested, the normal spleen cells and the spleen cells from the tumour bearing

week, three of the inoculated mice and three normal mice of the same age and sex were killed and tested simultaneously in the ordinary MC-test and also in the modified test involving preincubation of the spleen cells with and without Con-A. The table shows that when the spleen cells were tested in the ordinary MC-test without preincubation, only minor differences in target cell growth occurred when spleen cells from normal and tumour inoculated mice were added. However, after preincubation for 3 days a strong tumour inhibitory effect of the spleen cells from tumour inoculated mice was seen as compared with those of normal mice, when the cells were added in a concentration of 10^5 cells per well. This effect was found irrespective of whether the spleen cells were incubated with or without Con-A.

Stimulation of Tumour Cell Growth in vitro *after Addition of Spleen Cells from Mice Inoculated with Tumour*

Fig. 1 shows the result of a similar experiment. Again 30 mice were injected subcutaneously with tumour (3.5×10^5 cells/mouse). Three of these and three normal mice were killed every week. The spleen cells were incubated for 3 days before addition to tumour target cells. As seen in Fig. 1a, the spleen cells showed rapid and strong inhibitory reactions. 11 days after inoculation this inhibitory activity declined. The decline took place earliest with the lower spleen cell concentration and about a week later with the higher concentration. 25 days after inoculation, stimulatory activity occurred, i.e. the number of remaining target cells in wells incubated with spleen cells from mice inoculated with tumour was higher than the number of target cells in wells with spleen cells from normal animals. At the time of appearance of tumour (46 days), a period of inhibitory activity was again seen. When the spleen cells were incubated with Con-A (Fig. 1b), a similar picture resulted, but the initial inhibition was weaker and the subsequent stimulatory activity somewhat more pronounced. Again the lower spleen cell concentration caused stimulation earlier.

This behaviour led to the assumption that the stimulatory activity which occurred 18–39 days after inoculation was not due to the dominance of a certain stimulatory population of cells during that period, but was the result of a gradually diminishing number of reactive cells causing inhibition in high and stimulation in low concentrations.

In order to investigate this conception, tests were performed in which many different concentrations of spleen cells were added to the target cells. The results are shown in Table 2. Shifts are seen from no reaction to stimulation and then to inhibition with the addition of increasing concentrations of spleen cells. These results clearly show that the phenomenon of stimulation and inhibition is dose dependent, inhibition being dominant with the addition of high concentrations and stimulation with low concentrations of spleen cells. This relationship was seen independent of the time after tumour inoculation, but the concentration of spleen cells at which the inhibitory effect changed to stimulatory effect varied with the time after tumour inoculation and the test method used.

Analysis of the Kinetics of the Reaction between Spleen Cells and Target Cells in vitro

When the term stimulation or inhibition is used in the present study, it refers to an increase or decrease in the number of target cells remaining after the addition of spleen cells from tumour bearers relative to the number of target cells remaining after the addition of normal spleen cells. Therefore it was important to study the effect of both normal and tumour bearer spleen cells on target growth, compared with the growth of target

TABLE 2 *Addition to Tumour Target Cells of Different Concentrations of Spleen Cells from Mice Inoculated with Tumour*

Days after transplantation	Spleen cells added[a]	Con-a present during preincubation	Remaining number and percentage reduction/stimulation of tumour target cells after addition of doses of spleen cells: 0	1.5×10^3	3×10^3	6×10^3	12.5×10^3	25×10^3	50×10^3	75×10^3	100×10^3
43	N			121	151	148	159	175	257	305	260
		−	124	0% ns	1% ns	−8% ns	−16% ns	−45% ss	−21% ns	−2% ns	21% ss
	T			121	149	161	190	316	326	312	205
43	N			261	284	329	324	504	498	470	384
		+	226	−4% ns	−12% ns	−16% s	−32% ss	−8% ns	17% s	29% ss	27% s
	T			272	321	392	473	549	415	334	282

[a] N = spleen cells from normal mice
T = spleen cells from mice inoculated with tumour
ns = not significant
s = significant, $p < 0.05$
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effector cells would develop during this preincubation period, thus increasing the tumour killing effect.

Table 1 shows the results of an experiment in which 30 normal C3H mice were each given subcutaneously 10^6 viable tumour cells which had been thawed after nitrogen storage. This cryopreserved tumour also supplied the small amounts of tumour cells needed as target cells in the MC-test during the weeks of testing. The nitrogen storage thus made it possible to use the same tumour suspension as inoculum and as target cells. Even

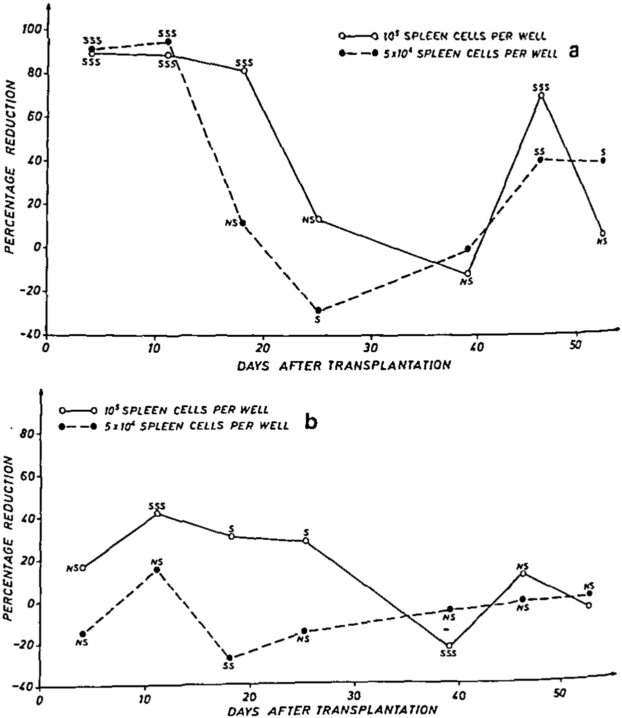


Fig. 1. Inhibitory and stimulatory effect of adding spleen cells from mice incubated with tumour target cells. The spleen cells were pre-incubated without (Fig. 1a) and with Con-A (Fig. 1b) before the addition to the target cells. Two concentrations of spleen cells were added, 10^5 and 4×10^4 per well. Percentage reduction is the relative reduction in number of target cells in experimental wells compared with wells with normal cells added. ns = no significant reduction, s = $p < 0.05$, ss = $p < 0.01$, sss = $p < 0.001$. (Students t-test).

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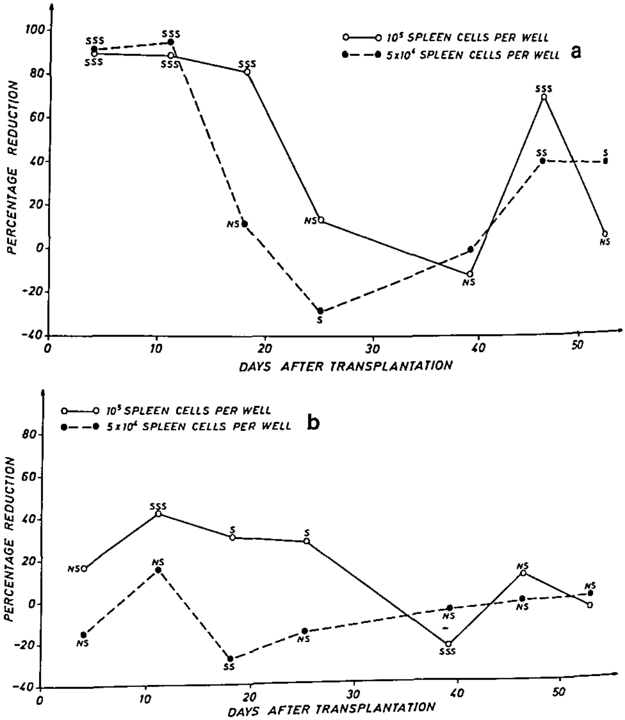


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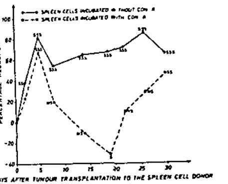


Fig 3 Inhibitory and stimulatory effect *in vitro* of spleen cells from animals inoculated with tumour 2–30 days earlier. The spleen cells were pre-incubated both with and without Con-A before the addition to the target cells Significance of the difference between experimental and normal cells group ns = not significant, s = $p < 0.05$, ss = $p < 0.01$, sss = $p < 0.001$

was palpable in the groups), all the animals were killed, the spleens removed and the spleen cells tested in the modified MC-test. The eight suspensions, plus a normal spleen cell suspension, were each mixed with tumour cells just prior to injection into nine groups of 10 mice Each mouse received 7 $\times$ 10^5 tumour cells and 50 $\times$ 10^5 spleen cells subcutaneously A 10th group was given tumour cells only. The tumour cells used for inoculation of donor mice and for injection together with spleen cells into recipient mice and for target cells were all taken from one original spontaneous C3H mamma carcinoma which had been stored in small aliquots in liquid nitrogen.

The result of this combined *in vitro* and *in vivo* experiment is shown in Figs. 3 and 4 *In vitro*, the result was almost the same as in the experiments in Fig. 1, showing an initial inhibitory period with maximum on day 5 followed by a period of declining activity. A maximum was reached on day 19, when cells preincubated with Con-A caused stimulation of target cell growth, and thereafter a period of inhibitory activity again occurred

When the spleen cells were transferred to recipients together with a tumour inoculum, the result was a general enhancement of tumour growth, as compared with the growth in the group given tumour plus normal spleen cells and in the group given tumour cells only The latter two groups had almost identical tumour growth The groups with the biggest enhancement of tumour growth were those given the spleen cells which were the most inhibitory *in vitro*, i e spleen cells from animals carrying 22–30 day-old tumour and from those carrying 5 day-old tumour

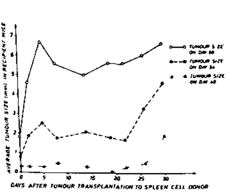


Fig 4 Tumour size in mice injected with tumour cells together with spleen cells from tumour bearing mice taken at different stages of tumour development Enhanced tumour growth in groups of mice having received a tumour inoculum plus spleen cells from mice injected with tumour cells 2–30 days previously The spleen cells were also tested *in vitro* (Fig 3) The figure shows the tumour size in the groups, measured at three different times, as a function of the time since the spleen cell donors were injected with tumour The average tumour diameter in the group which received tumour only was 0 3 mm on day 40, 1 0 mm on day 54, and 3 3 mm on day 68

DISCUSSION

In order to obtain the best approximation to the natural tumour phenomenon, an experimental system was used which involved transplantation of primary, spontaneously arisen C3H mamma carcinomas. Such tumours occurred with low frequency and were stored as cell suspensions in small aliquots in liquid nitrogen This method was preferred to serial passage *in vitro* or *in vivo* in order to avoid any changes in antigenicity or growth behaviour during the experiments.

The failure to detect *in vitro* reactivity of spleen cells from tumour bearing mice with the ordinary MC-test might be due to weak antigenicity of the tumours used It might also be due to inhibition of lymphoid cells by serum factors, but extensive washing of the cells before addition to target cells did not induce reactivity, as had been expected from the findings of others (3)

However, strong activity was obtained when the spleen cells were preincubated for 2–3 days *in vitro* before addition to target cells. Using this modification of the MC-test, reactivity was detected in spleens of mice after transplantation of any of the tumours used. A similar increased activity of lymphoid cells were also found by others (6, 9, 20) after one night of preincubation.

cells with no spleen cells added (medium control) during the 3 days of incubation.

The results of experiments in which the reaction between spleen cells and target cells was stopped on days 1, 2 and 3 after start are shown in Fig. 2 The growth of target cells without added spleen cells was dependent on the number of target cells seeded out Below a certain threshold number, there was progressive growth on day 1 and day 2, but a decline occurred on day 3. When the target cells were seeded out in numbers above the threshold there was a steady growth on day 3 also Addition of normal spleen cells to the target cells resulted in enhanced growth being greatest when 10^5 spleen cells were added. The addition of smaller numbers resulted in less enhancement. The addition of spleen cells from mice inoculated with tumour 5 days previously resulted in an opposite effect. Enhancement was seen when small numbers of spleen cells were added and the enhancing effect was greater than that of normal spleen cells, thus giving a relative stimulation, but this was not significant until day 3. However, the enhancing effect of spleen cells from tumour inoculated mice decreases rapidly as more spleen cells are added, and in the high concentrations where normal spleen cells have the biggest enhancing effect, spleen cells from tumour inoculated mice are the most inhibitory, leaving a number of target cells far below that of medium control. In this situation there is a relative inhibition of tumour cell growth, this being significant already on day 2 of culture. When lower numbers of target cells were seeded out, the number on day 3 was often diminished in medium control, as compared with wells with spleen cells added in concentrations as high as 10^5.

Enhanced growth of Tumours in vivo after Inoculation of Tumour together with Spleen Cells from Tumour Bearing Mice.

In order to investigate the influence of spleen cells from tumour inoculated mice on tumour growth *in vivo*, the following experiment was carried out During a period of 30 days, eight groups of three mice each received 4.4×10^5 tumour cells. 2 days after the last group was injected (before any tumour

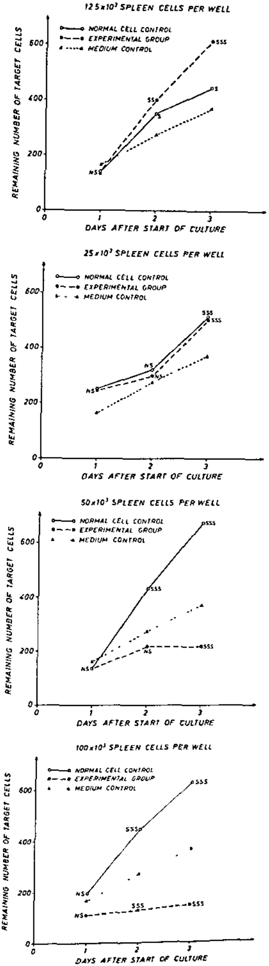


Fig. 2. Inhibitory and stimulatory effect of adding spleen cells from mice inoculated with tumour to tumour target cells. The spleen cells were pre-incubated 3 days without Con-A before addition to the target cells and thereafter the reaction was stopped on day 1, 2 and 3. Significance of differences between either experimental or normal cell group and medium control. ns = not significant, s = $p < 0.05$, ss = $p < 0.01$, sss = $p < 0.001$.

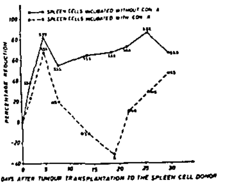


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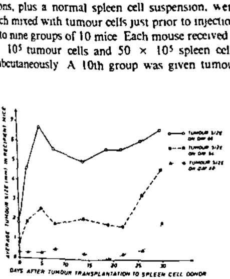


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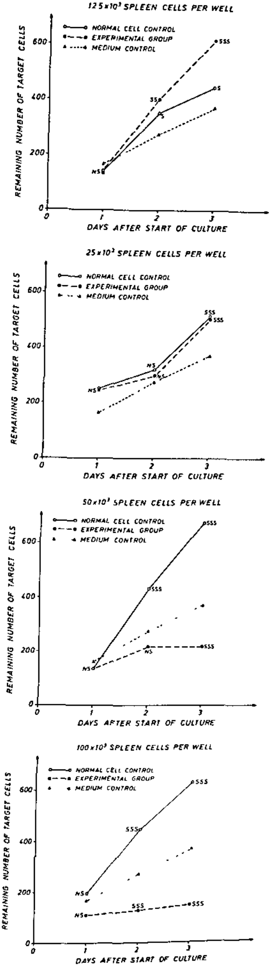


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Actually, recent observations by others point to a certain degree of non-specificity especially of the tumour enhancing activity of transferred spleen cells (18). It is therefore of importance to clarify whether the effects of the lymphoid system described by us are expressed only with syngeneic tumour target cells, and experiments are now being carried out including target tumours of different specificities.

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This dual behaviour *in vitro* of spleen cells from tumour bearing mice could be explained by assuming the existence of one population of reactive cells which are inhibitory when present in high and stimulatory in low concentrations. This suggestion would apply to the results obtained when testing spleen cells at different times after inoculation of tumour. An early period of strong inhibitory activities was seen 5–16 days after tumour transplantation. However, this activity declined or sometimes disappeared during the following weeks, often resulting in stimulatory activity. This stimulatory effect would thus not be the result of a new population of stimulatory cells, but simply the effect of the reactive cells being present among the spleen cells in such low concentrations that stimulation occurs.

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The tumour growth enhancement could also be due to transfer of supressor cells inhibiting an immune response against the tumour. Admittedly, the existence of suppressor cells is well documented in some different systems. These include the T cell mediated suppression of antibody synthesis (8) and the T and B cell mediated suppression of delayed type hypersensitivity (1, 19). Also in the tumour-host system suppressor cells may exist which modulate an immune response against the tumour, but the fact that a tumour grows in spite of the presence of an immune response against it may also be due to direct stimulatory capacity of the »immune« lymphoid cells (12, 14, 15).

Such stimulatory reactions may be the result of interactions between lymphocytes and weakly antigenic tumour cells. Strongly antigenic cells may induce cytotoxic reactions, as is the case during the rejection of highly antigenic tumours, while weakly antigenic tumours from a syngeneic source may induce an immune response which promotes the tumour growth This relationship was demonstrated by *Spärck* (14, 15) and the present results seem to give further support to the proposed immune stimulation hypothesis for tumour growth. It is conceivable that a strong immune response corresponding to a high concentration of reactive lymphoid cells inhibits the tumour, while the weak immune response, represented by a lower concentration of reactive cells, stimulates the tumour.

It must be emphasized, however, that the stimulatory and inhibitory effect reported may not be caused by immune reactions in the strict sense, i.e. the phenomena may be of a non-specific nature.

Actually, recent observations by others point to a certain degree of non-specificity especially of the tumour enhancing activity of transferred spleen cells (18). It is therefore of importance to clarify whether the effects of the lymphoid system described by us are expressed only with syngeneic tumour target cells, and experiments are now being carried out including target tumours of different specificities.

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BRIEF REPORT

Fcγ RECEPTORS IN HUMAN CHOROID PLEXUS

Harald Nyland and Roald Matre

Broegelmann Research Laboratory for Microbiology, and Department of Neurology, University of Bergen, Norway

Nyland, H. & Matre, R. Fcγ receptors in human choroid plexus Acta path microbiol scand Sect. C, 86 141–143, 1978

Cryostat sections of human choroid plexus adsorbed erythrocytes sensitized with IgG antibodies of human and rabbit origin No adsorption occurred when the erythrocytes were sensitized with $F(ab')_2$ or Facb fragments The reaction was strongly inhibited by intact IgG and by Fc fragments and not inhibited by Facb and $F(ab')_2$ or albumin These properties are similar to those of corresponding receptors in human placenta The presence of an Fcγ receptor in choroid plexus may be of significance for the transfer of IgG from blood to cerebrospinal fluid

Key words. Fcγ receptors, choroid plexus

H Nyland, Broegelmann Research Laboratory for Microbiology, MFH-bygget, N-5016 Haukeland sykehus, Bergen, Norway

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Cerebrospinal fluid (CSF) contains low concentrations of immunoglobulins as compared to serum (2, 13) The mechanism by which transfer of immunoglobulins from blood to CSF occurs is not well understood (4) Approximately half the CSF is produced from choroid plexus in the ventricles of the brain (8) At least some of the immunoglobulins are produced at these sites (5, 13) Epithelial cells in choroid plexus are connected with tight junctions, and transport of macromolecules across the epithelium is greatly restricted (blood-CSF-barrier) It has been suggested that albumin and gammaglobulins are actively transported through pinocytotic vesicles in the epithelial cells (2, 3, 10)

The data presented here indicate that human choroid plexus possesses receptors with specificity for the Fcγ portion of IgG These have aspects similar to the corresponding receptors in human placenta (7) which probably are of significance in the transfer of IgG from mother to foetus (1)

Materials and Methods

Tissue Human choroid plexuses were obtained at autopsies performed in the Department of Pathology 9–20 h after the death of patients suffering from cardiac infarction and without systemic disease Specimens from other organs were provided by the Department of Surgery Small pieces of tissue were rapidly frozen in liquid nitrogen and mounted on specimen holders for preparation of tissue sections. Pieces of tissues were also fixed in 4 % formaldehyde for histological investigation The remaining tissue was frozen embedded in PBS and wrapped in plastic bags for storage at −25° C

Sera Antisera to human immunoglobulins, albumin, C1q and C3 were purchased from Behringswerke, AG, Marburg, Lahn, West Germany. Antisera to sheep erythrocytes were produced by immunization of rabbits.

The sera were heated at 56° C for 30 min in order to inactivate complement Sera from patients with infectious mononucleosis were selected from material sent for routine serology.

Serum fractions Human IgG (fraction II, 16.5% solution) was purchased from AB Kabi, Stockholm, and human albumin from Behringwerke, AG. Rabbit IgG was obtained by ammonium sulphate precipitation of normal serum followed by gel filtration. Monoclonal human IgA and IgM were the same purified preparations as used previously (7)

IgG fragments. Rabbit $F(ab')_2$ and Facb, and human $F(ab')_2$ fragments were prepared in accordance with *Stewart et al* (11) Human and rabbit Fcγ fragments

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$$E \frac{1\%}{1\ cm}\ 280\ \mu m = 14.6.$$

Haemadsorption with tissue sections. The procedure with tissue sections was the same as described previously, using a closed chamber technique (12). The concavity of a microculture slide was filled with the indicator cells. A coverglass with a cryostat section of frozen tissue was placed on the slide with the section in the centre of the concavity pressed together and placed with the coverglass facing downwards to allow the cells to settle on the tissue sections. After 30 min at room temperature the slides were turned and left for indicator cells to detach from the glass and from non-reactive tissue to settle into the concavity of the microculture slides.

The indicator cells (EA) were prepared by sensitizing sheep erythrocytes (E) with rabbit IgG antibodies (A) in varying amounts expressed as agglutinating units. One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of 1% suspension of E. For some experiments, sheep E were sensitized with $F(ab')_2$ or Facb prepared from rabbit A, or with IgM antibodies from infectious mononucleosis sera or rabbit E sensitized with human natural IgG or IgA antibodies.

Haemadsorption was graded as 3+ when EA appeared in multilayers attached to the tissue, and as 2+ and 1+ when the tissue was covered by EA in tight monolayers or by single cells, respectively. Furthermore, the adsorbed EA appeared in a diffuse or focal pattern. Fixation and staining of the preparations were performed as described earlier (6).

Inhibition of haemadsorption. In these experimernts E were sensitized with the least amount of A giving a 3+ haemadsorption. EA were suspended in albumin or in the immunoglobulin preparations to be tested for inhibiting activity. The final concentrations of the preparations were 1 mg/ml. If the preparations contained agglutinins to E, they were exhausted by absorption with E before use. In some experiments the tissue sections were incubated with the antisera mentioned in various dilutions and washed in PBS before the EA were applied.

Results

Cryostat sections of human choroid plexus were tested with the various EA preparations sensitized to varying degrees from 4 – ½ agglutinating units. With strongly sensitized IgGEA (4 and 2 agglutinating units), almost the whole sections were covered. Using weaker sensitization, the EA attached in a focal pattern and apparently mainly to the epithelial tissue. No significant differences in reactivity with specimens from 8 different human choroid plexuses were detected. The EA were properly sensitized as shown by the reaction with the Fcγ positive areas of liver, spleen and placenta (7, 12). Rabbit E sensitized with human IgG were also adsorbed, whereas E prepared with human IgM or salivary IgA were not. These results indicate that the human choroid plexus contains receptors for IgG.

TABLE 1. *Haemadsorption of EA[a]) to Tissue Sections Effect of Rabbit IgG and IgG Fragments in the EA Suspension*

Type of tissue	Saline	EA suspended in IgG or IgG fragments[b]) IgG	$F(ab')_2$	Fc	Facb
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Placenta	3+	–	3+	–	3+
Spleen	3+	–	3+	–	3+

a) EA = sheep erythrocytes sensitized with rabbit IgG
b) Final concentration, 1 mg/ml.

The preferential binding of IgG was further substantiated by the results obtained in inhibition experiments. Rabbit and human IgG (Table 1) inhibited the haemadsorption to choroid plexus, whereas human IgA, IgM and albumin did not, even at a concentration of 16 mg/ml. The haemadsorption was dependent on complete IgG molecules, as shown by the lack of reactivity with E sensitized by $F(ab')_2$ or Facb fragments of rabbit A, even in agglutinating amounts. In addition, neither rabbit $F(ab')_2$, Facb nor human $F(ab')_2$ fragments (4 mg/ml) inhibited the adsorption of IgGEA, in contrast to the marked inhibition obtained with Fcγ fragments (Table 1).

Sections of choroid plexus were also incubated with rabbit antiserum to human immunoglobulins, C1 Q, C3 and albumin, before incubation with IgGEA. Either no inhibition was found or there was inconstant inhibition at low dilutions

The influence of temperature on the haemadsorbing activity was investigated by performing the test at 4°C, 20°C and 37°C. No difference in the degree of haemadsorption was recorded. Formaldehyde fixed and haematoxylin and eosin (H&E) stained sections of the choroid plexuses investigated showed normal structure with epithelial, endothelial and some stromal cells, and no infiltration of monocuclear cells.

Discussion

The results obtained show that human choroid plexus possesses a receptor for the IgG molecule. The results obtained in experiments with various fragments of human and rabbit IgG indicated strongly that the receptors reacted with the Fc portion of IgG molecules. The lack of binding of Facb suggests that the Cγ3 region is of importance for the binding. These properties of the choroid plexus receptor are similar to those of the placenta Fcγ receptor. Further similarities of the reaction

were *indepence* of temperatures between 4°C and 37°C and the lack of blocking with antisera to immunoglobulins, C3 and albumin. The pattern of binding indicated that the receptors are located at the epithelial tissue. In order to obtain a more precise localization of the Fcγ receptors, experiments with immune complexes of horseradish peroxidase (HRP)-rabbit IgG anti-HRP are in progress.

The presence of Fcγ receptors in human choroid plexus may be of significance for the transport of IgG from blood to the cerebrospinal fluid. At least some of the IgG is transferred through the choroid plexus (5). The presence of pinocytotic vesicles in the epithelial cells of the plexus indicates the existence of active transport mechanisms for macromolecules (2, 3). The lack of IgA in cerebrospinal fluid from brain ventricles (13), in spite of a similar molecular weight with IgG, gives further support to the assumption that the Fcγ receptor may play a role in the transfer of IgG from blood to the cerebrospinal fluid.

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Sections of choroid plexus were also incubated with rabbit antiserum to human immunoglobulins, C1q, C3 and albumin, before incubation with IgGEA Either no inhibition was found or there was inconstant inhibition at low dilutions

The influence of temperature on the haemadsorbing activity was investigated by performing the test at 4°C, 20°C and 37°C No difference in the degree of haemadsorption was recorded Formaldehyde fixed and haematoxylin and eosin (H&E) stained sections of the choroid plexuses investigated showed normal structure with epithelial, endothelial and some stromal cells, and no infiltration of monocuclear cells

Discussion

The results obtained show that human choroid plexus possesses a receptor for the IgG molecule. The results obtained in experiments with various fragments of human and rabbit IgG indicated strongly that the receptors reacted with the Fc portion of IgG molecules. The lack of binding of Facb suggests that the Cγ3 region is of importance for the binding. These properties of the choroid plexus receptor are similar to those of the placenta Fcγ receptor. Further similarities of the reaction

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PROTECTIVE ANTITOXIC CHOLERA IMMUNITY IN MICE: INFLUENCE OF ROUTE AND NUMBER OF IMMUNIZATIONS AND MODE OF ACTION OF PROTECTIVE ANTIBODIES

S LANGE and J HOLMGREN

Institute of Medical Microbiology, University of Goteborg, Goteborg, Sweden

Lange, S & Holmgren J Protective antitoxic cholera immunity in mice Influence of route and number of immunizations and mode of action of protective antibodies Acta path microbiol scand Sect C, 86 145–152, 1978

An adult mouse model has been elaborated for studies of experimental cholera (*Vibrio cholerae* enterotoxin-induced intestinal secretion) and protective antitoxic immunity in either ligated small bowel loops or intact small intestine Mice of different inbred strains varied markedly in intestinal susceptibility to toxin, C57Bl being the most sentive strain tested Fluid accumulation started within 1 h after the inoculation of toxin and was maximal after 5 h, whereafter recovery gradually ensued The dose-response curve was sigmoid, the ED_{50} of crude toxin being equivalent to about 0 1 µg purified toxin/cm in the loops and 0 3 µg/cm in the nonligated intestine Two peroral (p o) immunizations induced significant protective immunity which increased markedly after two further immunizations by the same route Additional p o immunizations did not appreciably enhance the protective immunity any further Intravenous (i v) vaccination had to be repeated more than 5 times before intestinal immunity could be observed No correlation between serum antitoxin titers and protective immunity was found Electron microscopic examination revealed that whereas peroxidase-coupled cholera toxin bound tightly to intestinal microvilli from unimmunized or 5-times i v immunized mice it did not bind to the microvilli of p o immunized animals The data thus suggest that the protective immunity is mediated exclusively by locally produced antibodies which prevent the binding of toxin to the gut epithelium

Key words *Vibrio cholerae* enterotoxin, experimental cholera, mouse model, protective immunization, local immunity, peroxidase-conjugated cholera toxin

S Lange, Institute of Medical Microbiology, University of Goteborg, Guldhedsgatan 10, S-413 46 Goteborg Sweden

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The enterotoxin of *Vibrio cholerae* has been shown to induce experimental diarrhoea when injected intestinally in several animal species including dogs (15), rabbits (12), hamsters (3), chinchillas (4) and rats (1) The mouse would represent an especially suitable test animal for several reasons. It is cheap, easy to handle, and available in several genetically homogenous, inbred strains. Suckling mice have recently been successfully used in analysing the diarrheogenic effect of live *V. cholerae* as well as cholera toxin (2, 5, 11, 16, 17) Although useful for passive immunization studies of protection by antiserum or immune milk (5, 11, 16, 17), the infant mouse model is obviously not suitable for investigations of the intestinal response obtained during the active immunization. *Fujita & Finkelstein* described a single loop-intestinal assay in the adult mouse which they claimed to be useful for estimation of toxin induced diarrhoea as well as of

RESULTS

Experimental cholera in intact and ligated small intestine Four inbred strains of mice were tested for intestinal secretory responsiveness to *V cholerae* enterotoxin Graded doses of crude cholera toxin were injected into ligated small bowel loops or the intact small intestine and the fluid accumulation registered 5 h later. At low toxin doses the response differed markedly between the mouse strains, C57Bl being most and DBA least sensitive (Table 1). The maximal intestinal secretory capacity, on the other hand, did not seem to differ appreciably between the strains, since C57Bl and DBA produced a similar fluid volume per cm intestine after challenge with a high dose of toxin in the loop system (Table 1) The subsequent studies were performed in C57Bl mice

TABLE 1 *Different Responsiveness of Mice of Various Inbred Strains to Intestinal Challenge with Cholera Toxin*

	Fluid accumulation (mg weight increase/cm intestine[a])				
	Loops			Intact intestine	
	Crude toxin challenge, mg				
Mice	1 5	5	100	25	40
C57Bl	47	110	162	9 6	16
A/Sn	29	82	NT[b]	6 3	NT
Balb/c	23	28	NT	0 7	NT
DBA/1	15	20	175	3 6	5 5

[a] each value is the mean of 4 animals
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To ascertain that the intestinal fluid accumulation induced by the crude cholera toxin was an enterotoxin specific effect, a high dose of toxin was mixed with specific antiserum to purified cholera enterotoxin or incubated at 56° C for 30 min before being used for challenge. These treatments totally abolished the capacity of the crude toxin to induce intestinal fluid secretion (Table 2)

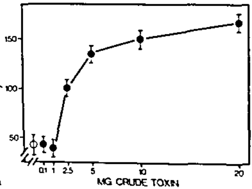
TABLE 2 *Specificity in Action of Crude Cholera Toxin on Intestinal Fluid Accumulation*

Challenge	Weight (mg/cm) Intestinal loop	Intact small intestine
Toxin[a]	135 ± 8[c]	47 9 ± 3
Toxin, 56° C for 30 min	27 8 ± 4	26 3 ± 2
Toxin + antiserum[b]	28 2 ± 3	25 1 ± 3
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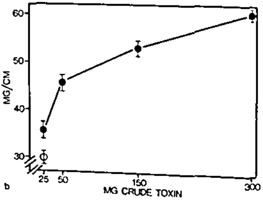
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The dose-response relationship was tested in ligated loops as well as in the non-ligated intestine. In the loops half-maximal fluid accumulation was produced by about 2.5 mg (0 4 mg/cm) of the crude toxin (Fig 1a) In the intact intestine ED_{50} was about 50 mg, i e. 1 mg/cm (Fig. 1b). Control experiments in the loop assay with purified cholera toxin gave a very similar dose-response curve, ED_{50} being 1 0 µg (not shown).

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a



b

Fig 1 Dose-response relationship between intestinal toxin challenge and fluid accumulation in (a) ligated loops and (b) intact intestine The relative weight (mg/cm) of control intestine (saline-injected loops or intact intestine (o), and intestine challenged with various toxin doses (●) are shown, the difference representing the fluid accumulation Mean values ± SEM (vertical bars) of 5 animal groups are shown

protective antitoxic immunity (8). In contrast, *Lepot & Banwell* recently classified the adult mouse to be unresponsive to intestinal toxin challenge (13). In this study we have further explored the possibilities of using mice for investigations of experimental cholera and antitoxic immunity. The specific objectives were: 1) to define conditions for production of experimental diarrhoea by cholera toxin in adult mice, and 2) to evaluate the protective antitoxic immunity after enteral and parenteral immunization.

MATERIALS AND METHODS

Mice. Inbred 8 to 12 week old mice of four strains (C57B1/6J, DBA/1J, A/Sn, Balb/c) were used. Within each experiment the animals were of the same sex and differed no more than 10 days in age and 5 g in weight, precautions which are important to diminish individual variation.

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Immunizations Intravenous (i.v.) injections were given in the lateral tail vein in a volume of 0 1 ml Control mice were similarly injected with phosphate-buffered saline (PBS, 0.05 M phosphate, 0.14 M NaCl, pH 7.2) Peroral (p o) immunization was obtained by instillation via a baby feeding tube marked 4 cm from the end under light ether anesthesia. The toxin, 0 25 ml, was mixed with 0.25 ml of $NaHCO_3$ (100 mg/ml, pH 8 5) before the instillation The bicarbonate was shown to neutralize the gastric acid for about 1 h without affecting the toxin Mice given a total of 0 5 ml bicarbonate – PBS solution were used as controls

Assays of Experimental Cholera and protective Immunity

a *Ligated loops* Mice, fasted for 24 h but not deprived of water, were anesthesized with ether, the abdomen opened, and the small intestine ligated in two 6–8 cm long loops starting approximately 10 cm from the duodenum The loops were injected with different doses of toxin in a volume of 0 2 ml, and the abdomen closed with agraffes Control loops were not done because a large number of experiments had shown that loops injected with PBS never revealed any positive reactions. Food and water were excluded and when not specified otherwise the mouse was sacrificed after 5 h The length of the loop was measured, and the loop with the accumulated fluid weighed This gave considerably greater accuracy than volumetric measurement of the secreted fluid, since the total volume was small

b. *Intact small intestine.* Experimental cholera was also produced in the non-ligated mouse intestine. After the abdomen had been opened 0.5 ml toxin was injected into the upper duodenum. The animals, deprived of food and water, were usually sacrificed after 5 h The small intestine was carefully freed from the mesenteric tissue, the length measured, and the intestine with accumulated fluid weighed.

c. *Protection by immunization.* The fluid accumulation induced by cholera toxin in the ligated or non-ligated intestine was found to be dose-dependant when estimated in non-immunized as well as immunized animals The dose-response curve was typically S-shaped in both systems and the ED_{50} value, i e. the toxin dose giving half-maximal fluid accumulation, was interpolated between the upper and lower asymptote The protective effect of immunization – the protective factor – was estimated as the ratio between the ED_{50} values of the vaccinated and concurrently tested non-immunized control groups

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Binding of peroxidase coupled CT to intestinal mucosa Coupling of peroxidase to purified cholera toxin was done as described elsewhere (*Lange, S , Hansson, H. A & Nygren, H* , to be published). Radial immunodiffusion (Mancini) tests revealed that the slope for concentration vs halo precipitate area was similar for coupled and native toxin, indicating that the coupling procedure had not denatured the toxin The labelled toxin was biologically active producing fluid accumulation in the mouse loop assay when tested in a dose of 4 μg Small pieces of intestinal tissue from vaccinated or control mice were incubated at 37° C for 30 min with 6 μg/ml of peroxidase-coupled cholera toxin in TC 199 medium (Flow laboratories, Svenska AB, Solna) The specimens were fixed with 3% glutaraldehyde in 0 15 M cacodylate-buffer, incubated with diamino-benzidine substrate and embedded in Epon. Thin sections were examined in an electron microscope

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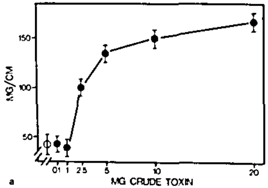
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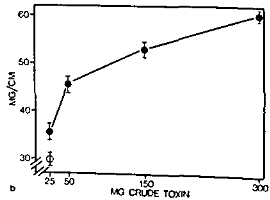
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a



b

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TABLE 2 *Specificity in Action of Crude Cholera Toxin on Intestinal Fluid Accumulation*

Challenge	Weight (mg/cm) Intestinal loop	Intact small intestine
Toxin[a]	135 ± 8[c]	47.9 ± 3
Toxin, 56° C for 30 min	27.8 ± 4	26.3 ± 2
Toxin + antiserum[b]	28.2 ± 3	25.1 ± 3
PBS	26.2 ± 4	27.6 ± 2

[a] Toxin challenge in the loops were 5.0 mg, and in the intact small intestine 50 mg (crude toxin)
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The dose-response relationship was tested in ligated loops as well as in the non-ligated intestine. In the loops half-maximal fluid accumulation was produced by about 2.5 mg (0.4 mg/cm) of the crude toxin (Fig 1a) In the intact intestine ED_{50} was about 50 mg, i e 1 mg/cm (Fig. 1b). Control experiments in the loop assay with purified cholera toxin gave a very similar dose-response curve, ED_{50} being 1.0 µg (not shown).

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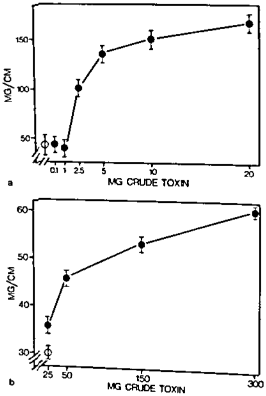


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TABLE 3 *Protective Immunity and Serum Antibody Titres after Enteral or Parenteral Immunization with Cholera Toxin*

Route and number of immunizations[a]	Protective factor: Ileal loop	Protective factor: Intact intestine	Antitoxin serum titre[b]
Peroral			
1	1	1	<1
2	2	3	1024
4–9	7–11	>5	1024
Intravenous			
1	1	1	64
2	1	1	2048
4–5	1	1	32768
9	2	NT	16384

[a] Mice were immunized with 1 µg CT i v or 5 µg CT p o The first booster dose was given 10 days after the primary injection, subsequent immunizations were given every sixth day

[b] Tested 6 days after the primary injection and 4 days after the booster dose. The protective factor refers to the ratio between the ED_{50} values of immunized and concurrently challenged control animals The antitoxin titres were determined by passive haemolysis NT, not tested

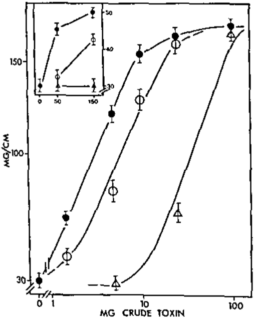


Fig 3 Demonstration of protective antitoxic immunity after repeated enteral immunization Groups of mice were given two or four p o immunizations and their resistance to intestinal challenge with graded doses of toxin evaluated 4 days after the last immunization using either the ligated loop assay (main figure) or the intact intestine system (insert) ●—● represent intestinal weights of unimmunized controls before and after challenge, ○—○ the post-challenge weights of mice immunized twice p o, and △—△ the weights of mice given 4 p o immunizations The values are the means ± SEM of 5 animals

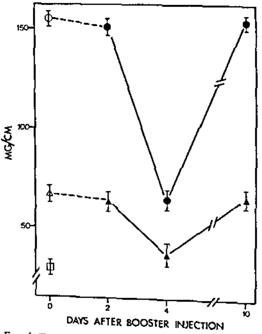


Fig 4 Time course of protective antitoxic immunity. The mice were immunized 4 times p o, and then at the indicated times challenged with 25 mg (●) and 5 mg (▲) of crude cholera toxin in intestinal loops Open symbols indicate the effect of challenge on non-immunized mice, □ is the weight of non-challenged intestine It is seen that protection is maximal 4 days after immunization, while no protective immunity is noted after 2 or 10 days

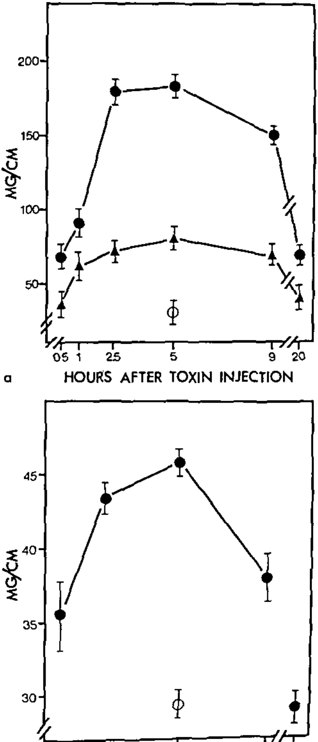


Fig 2 Kinetics of cholera toxin induced intestinal weight increase (fluid accumulation) in (a) ligated loops after challenge with 25 (●) or 3 (▲) mg of crude toxin, and (b) intact small intestine after challenge with 100 mg of crude toxin (●) O re the control values, i.e. the weight of saline-injected loops or intact intestine. Mean values ± SEM (vertical bars) of 5 animal groups are presented.

ight (including the lumen fluid) gradually declined to be normal within about 24 h in the loops (Fig 2a) and 15–20 h in the non-ligated intestine (Fig. 2b) From the data in Fig. 2 and experiments in which the rate of fluid absorption in the normal small intestine was estimated to 0.53 mg/cm/min it can be calculated that active secretion probably continued from the toxin-stimulated intestine for the whole study period (20 h). In order to evaluate to what extent the faster decrease of fluid in the intact intestine than in the loops was due to peristaltic transport of fluid to the colon, groups of mice in which the ileum had been ligated at its entrance in the colon ascendens were studied. No difference in fluid accumulation between the intact and the distally ligated small intestine was found after 5 h, but after 16 h the ligated intestine contained 30 per cent more fluid than the non-ligated one.

Protective effect of immunization. The two models of experimental cholera were used to evaluate the development of protective immunity following immunization of the mice with toxin antigen. Single and repeated immunizations were given parenterally (i.v.) or enterally (p o.) and protection tested 4 days after the immunization.

The i.v. immunizations consisted of 1 µg of purified cholera toxin in each injection, since preliminary experiments had shown that this dose produced maximal levels of serum antibodies. However, neither a single nor up to five times repeated i.v. immunization gave rise to any significant change in responsiveness to intestinal challenge with cholera toxin (Table 3). This lack of protection was observed in both the ligated-loop and the intact intestine models. Nine immunizations gave rise to a significant although still low level of immunity, ED_{50} being about 2-fold increased (Table 3).

A single p.o. immunization with 5 µg of the purified toxin was ineffective. Booster immunization, however, induced a significant inhibition of the fluid accumulation after challenge with toxin both in the ileal loop and the intact small intestine systems (Fig. 3). The magnitude of the protection was relatively small, though, the protective factor being about 2-fold in the loop and 3-fold in the intact intestine (Table 3) A higher antigen dose (15 µg) gave no further protection. In contrast, a striking increase in protective immunity was attained by two further 5 µg-doses (Fig. 3). Additional immunizations did not further enhance the magnitude of protection (Table 3).

Time course of immunity. The kinetics of the protective immunity obtained by 4 p o. immunizations was studied in the ileal loop system (Fig. 4) Inhibition of intestinal secretion after challenge with

fully reproduced the action of the crude material. Subsequent studies have shown that similar intestinal responses as those obtained with cholera toxin can be induced with heat-labile *Escherichia coli* enterotoxin, prostaglandin E_1 and dibuturyl cyclic AMP (*Holmgren, Lange & Lönnroth*, to be published)

The rate of net fluid secretion was maximal from 1 to 3 h after toxin challenge, while the maximal fluid accumulation was noted after 5 h. Thereafter the absorptive capacity of the gut apparently exceeded the secretion leading to a decrease in accumulated fluid In the loops this decrease was linear with time In the intact intestine, on the other hand, the fluid decrease rate was considerably faster from 5 to 9 h after toxin challenge than thereafter This and the demonstrated fluid preserving effect of terminal ligation of the ileum indicate that peristaltic contractions contribute significantly to the removal of fluid as long as the intestine is distended It can be calculated that if the absorptive capacity of the small intestine were unaffected by toxin, the observed rate of fluid decrease in the loops would require an almost constant active secretion during the period from 5 to 20 h However, cholera toxin is known to impair absorption as well as to stimulate active secretion (6) Thus, it seems likely that the secretory rate decreases gradually after 5 h but is still significant as late as 20 h after the application of toxin

In previous investigations the adult mouse has been described both as totally resistant (13) and as well responsive (8) to the diarrheogenic action of cholera toxin The present results might elucidate the basis for this discrepancy by showing marked differences in responsiveness between various mouse strains. Although most or all strains appear to give a similar response to high toxin doses the differences are pronounced at lower doses to which certain strains, including DBA and Balb, are resistant while others, like C57Bl, are sensitive

Protective intestinal immunity to cholera toxin has mainly been studied in rabbits and dogs Parenteral vaccination of dogs induces protection probably due to an influx to the gut of antitoxin antibodies from serum (14), while the rabbit intestine after parenteral vaccination apparently is protected by a combination of serum antibodies and intestinally synthesized immunoglobulin (10) Oral or enteral vaccination with toxin or toxoid does not protect dogs (14), while such immunization of rabbits gives significant immunity probably mediated by immunoglobulin produced by intestinal plasma cells (10) In the mouse, as shown in the present studies, a significant protective intestinal immunity is obtained much more easily by enteral than by parenteral immunization. Only after 9 i v. immunizations with a highly immunogenic dose of toxin there was any measurable protection against intestinal toxin challenge, whereas 4 p o. immunizations gave protection of much higher magnitude. The immunity seems to be of short duration with a peak after 4 days There was no correlation between the resistance to cholera toxin challenge and the serum levels of antitoxic antibodies indicating that the intestinal protective immunity was exclusively due to a local immune response. The present electron microscopic studies show that peroxidase-labelled cholera toxin is unable to attach to the intestine of immune mice, in contrast to the heavy toxin staining of the intestinal microvilli of non-immune animals It may therefore be concluded that protective antitoxic cholera immunity in the mouse is mediated mainly or exclusively by antibodies synthesized by intestinal plasma cells, and that these antibodies act by combining with the toxin molecule at the mucosal surface or in the lumen thus preventing binding of toxin to the intestinal G_{M1} ganglioside receptors.

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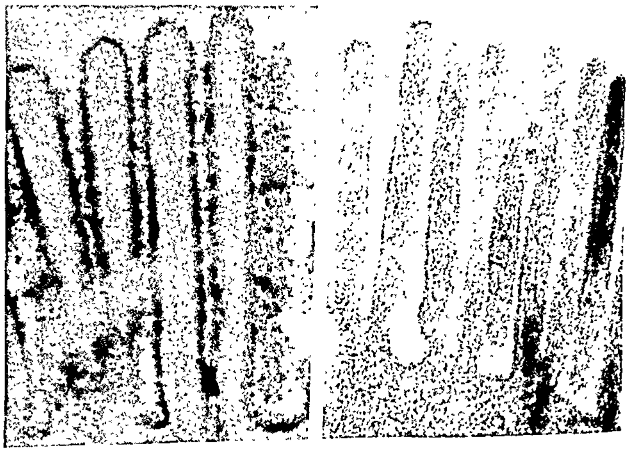


Fig 5 Electron micrograph showing *in vitro* attachment of peroxidase-coupled cholera toxin to intestinal microvilli of mice immunized 5 times i v (left) but not to microvilli of mice given 5 p o immunizations resulting in protection (right) The intestinal staining pattern of the i v vaccinated animals does not differ from that of control mice (15000 × magnification)

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Acta path microbiol. scand Sect. C, 86: 153–158, 1978.

FREEZING OF RAT LYMPHOCYTES

V. The Effect of Suppressor Cells in Phytohaemagglutinin-Stimulated Spleen Cell Cultures before and after Freeze-Thawing

EINAR HEM

Laboratory for Haematology and Lymphology Norsk Hydro's Institute for Cancer Research Montebello, Oslo 3, Norway

Hem, E Freezing of rat lymphocytes. V. The effect of suppressor cells in phytohaemagglutinin-stimulated spleen cell cultures before and after freeze-thawing Acta path microbiol. scand. Sect. C, 86: 153–158, 1978

Spleen lymphocyte proliferation, as measured by ^{3}H-thymidine incorporation induced by phytohaemagglutinin (PHA), was increased after freeze-thawing with 10% dimethyl sulphoxide. Depletion or intoxication of macrophages in fresh spleen cell preparations also increased lymphocyte proliferation in response to PHA On the other hand, freezing of macrophage-depleted spleen cell suspensions lowered ^{3}H-thymidine uptake of stimulated cultures At concentrations above 3%, macrophages added to cultures of fresh purified lymphocytes showed a dose-dependent inhibitory effect on the PHA response, and fresh macrophages were more inhibitory than frozen-thawed macrophages. Purified lymphocytes mixed with 10% macrophages showed a higher response after freeze-thawing It is concluded that macrophages suppress the lymphoproliferative response to PHA in rat spleen cell cultures, and that these macrophages are more sensitive than lymphocytes to the present freeze-thaw process.

Key words Suppressor macrophages, rat spleen, freezing

Present address E Hem, Surgical Department II, Ullevål Hospital, Oslo 1, Norvay

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In vitro proliferative responses in the rat to non-specific mitogens or allogeneic cells have demonstrated that spleen cell preparations give lower responses than cells from blood, thoracic duct and lymph nodes (11, 15, 25). This has been shown to depend on the presence of suppressor cells in the spleen rather than to a lack of cells capable of the response (8, 9, 15, 16) The nature of these cells has remained more obscure An understanding of this phenomenon might be of importance in connection with freeze-storage of lymphoid cells. In a previous study, freeze-thawing of rat spleen cells with dimethyl sulphoxide (DMSO) as a cryoprotectant introduced an unexpected elevation of labelled thymidine uptake of cultures stimulated with phytohaemagglutinin (PHA) (11) This was interpreted as being caused by a deleterious effect of freeze-thawing on suppressor cells present in the spleen, since the phenomenon was absent in lymph node preparations. The purpose of the present study was to examine in more detail the nature and effect of freezing on the suppressor activity present in the spleen cell cultures.

MATERIALS AND METHODS

Animals Inbred, locally-grown Hooded rats 8–11 weeks old were used Rats of either sex were used in each experiment.

Antigen administration Washed, packed sheep red blood cells (SRBC) from the same sheep were injected into rats in 2 ml volumes via an exposed femoral vein 18–24 h before sacrifice

Cell preparation Spleens were removed aseptically from anaesthetized, exsanguinated rats and teased through a stainless steel mesh into medium RPMI 1640 with Hepes buffer (Gibco-Biocult,Glasgow, Scotland) containing 100 µg streptomycin and 100 IU penicillin per ml Clumps

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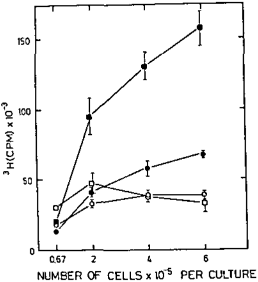


Fig 2 Dose response curves of cell proliferation by fresh (-O-) and frozen-thawed (-●-) normal spleen cells stimulated with PHA, and fresh (-□-) and frozen-thawed (-■-) stimulated spleen cells from rats injected i v with 2 ml packed SRBC 24 h before sacrifice Vertical bars on mean values represent ± 1 standard deviation of triplicate cultures

of fresh cells with carragheenan gave a much higher ^{3}H-thymidine incorporation than fresh cells without carragheenan (Fig. 4). When cells were frozen-thawed, the finding was the opposite viz. cells with carragheenan gave a lower response than cells without carragheenan, except for the highest cell concentration.. The difference between cultures of frozen-thawed cells with and without carragheenan was not as great as with fresh cells.

5. Effect on PHA response of addition of fresh and frozen-thawed macrophages to cultures of non-adherent normal spleen cells (NASC)

Fresh column purified spleen cells were cultured at 2 × 10^5 cells per well with increasing concentrations of fresh and frozen-thawed peritoneal macrophages and stimulated with PHA. Figure 5 shows that lower concentrations of macrophages (<3%) seemed to have some stimulatory effect on ^{3}H-thymidine uptake by the PHA-stimulated purified spleen cells. However, by increasing the concentration of macrophages in the cultures, a dose-dependent inhibitory effect could be demonstrated. At all doses, the inhibition by fresh macrophages was stronger than the inhibition by frozen-thawed macrophages

greater with ISC Therefore ISC was used in the following three experiments For both SC and ISC, the difference between fresh and frozen-thawed cells was most pronounced at higher cell numbers. It should be noted that the differences between frozen-thawed SC and ISC are not due to differences in the lymphocyte populations, since column purified spleen cells from both sources showed similar high responses (own results not shown) After freeze-thawing, the percentages of esterase positive cells recovered in suspensions of SC and ISC were 7–8% and 3–4%, respectively

3. Effect of freeze-thawing on PHA response of ISC after removal of adherent or phagocytic cells

Both removal of adherent and phagocytic cells markedly elevated the ^{3}H-thymidine uptake in stimulated cultures of fresh cells (Fig 3) Freeze-thawing of these non-adherent cells or phagocyte depleted cells lowered the isotope uptake in response to PHA At higher cell concentrations, it was still far above that of untreated fresh ISC

4 Effect of intoxication of macrophages by carragheenan on PHA response of cultures of fresh and frozen-thawed ISC.

Carragheenan was added to PHA stimulated cultures of fresh and frozen-thawed ISC. Cultures

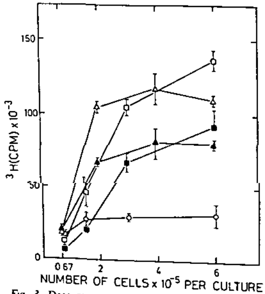


Fig. 3 Dose response curves of cell proliferation by PHA-stimulated spleen cells from rats injected i v. with SRBC (-O-) fresh untreated cells, (-Δ-) fresh and (-▲-) frozen-thawed column purified cells, and (-□-) fresh and (-■-) frozen-thawed cells from spleen cell suspension depleted of phagocytic (treated with carbonyl iron and magnet) cells Vertical bars on mean values represent ± 1 standard deviation of triplicate cultures

were allowed to settle for 10 min, after which the supernatant was carefully removed, washed three times, resuspended and counted in an electronic cell counter. Lymph nodes (cervical, brachial and mesenterial) were teased directly in the medium and further handled as for spleen cells. Cultures and incubations were performed in RPMI 1640 with bicarbonate buffer and antibiotics as above.

Peritoneal cells (PC) Killed rats were injected intraperitoneally with 20 ml phosphate-buffered saline, and after gentle abdominal massage 16–18 ml of cell suspension was withdrawn. The cells were then washed and counted as for the lymphocytes.

Histochemical demonstration of macrophages/monocytes. This was performed by the neutral esterase staining method as described by *Schmalzl & Braunsteiner* (22). Lymph node suspensions contained 2–4 %, spleen cell preparations from both normal and immunized rats 15–20 %, and peritoneal washings 65–80 % esterase positive cells.

Removal of adherent cells. This was performed according to the procedure outlined by *Bash & Waksman* (3). Three ml spleen cells (5–10 × 10^7) in RPMI 1640 with 10 % fresh rat serum (FRS) were poured over a glass wool column (1 gram glass wool in a 10 ml syringe, preincubated with the 10 % FRS containing medium) and incubated for 45 min at 37°C. Non-adherent cells were then eluted from the column with 30 ml warm (37°C) medium. Usually 40–50 % of the cells could be recovered from the columns, and numbers of esterase positive cells were reduced to <0 5 %

Removal of phagocytic cells. Spleen cells (1 × 10^8) in 10 ml RPMI were incubated with 0.6 g carbonyl iron powder (Grad SF, GAF Svenska AB, Stockholm, Sweden) and rotated vertically (6 rotations per min) for 45 min at 37°C. After incubation, the non-phagocytic cells were removed by placing a powerful magnet below the tube and aspirating the supernatant carefully. Some 50 % of the cells were recovered after this procedure, and the numbers of esterase positive cells were reduced to about 1 %

Macrophage intoxication Carragheenan (Sigma Chemical Co, St Louis, Mo, USA) was used as a drug specifically cytotoxic for macrophages and not lymphocytes (1, 5) and was added at the initiation of the cultures. The final concentration of carragheenan in the cultures was *1 mg/ml*

Freeze-thawing. Freezing was performed in a Revco ultralow freezer as described previously (12). Briefly the freezing suspension consisted of 65 % RPMI 1640 with Hepes buffer, 25 % rat serum and 10 % dimethyl sulphoxide (DMSO) was added slowly and equilibrated for 10 min at room temperature. Five ml samples in Teflon/Kapton bags were cooled at 2°C/min to 0°C and at 6°C/min to −60°C and then stored in liquid nitrogen (−196°C) for 10 min. Thawing was performed in waterbath at 37°C, and 45 ml RPMI 1640 with 10 % serum was added slowly at room temperature before a final wash at *100 g* for 5 min.

Cell cultures. Cell cultures were performed in microtitre plates as described previously (12). Each well contained 2 × 10^5 lymphoid cells in 150 µl medium RPMI 1640 with bicarbonate buffer, antibiotics and 5 % fresh rat serum. They were stimulated with 0.3 % PHA (Phytohaemagglutinin-P, Difco, Detroit, Michigan). Cultures were pulsed with 1.25 µCi ^{3}H-thymidine for the last 4 h of incubation and harvested at 48 h on a Skatron Multiple Cell Culture Harvester.

RESULTS

1. Effect of freeze-thawing on PHA response of lymph node cells:

For cell numbers between 5 × 10^4 and 4 × 10^5 fresh or frozen-thawed cells per well, there was an almost linear correlation between cell dose and ^{3}H-thymidine uptake in stimulated cultures (Fig. 1) Response to PHA was lower in the frozen-thawed than in fresh cultures.

2. Effect of freeze-thawing on PHA response of normal spleen cells (SC) and spleen cells from rats injected with SRBC (ISC):

The isotope incorporation by fresh spleen cell cultures was far below that of lymph node cell cultures (Fig. 2). Except for the lowest cell dose, there was a significantly higher uptake in cultures of frozen-thawed cells, and this difference was even

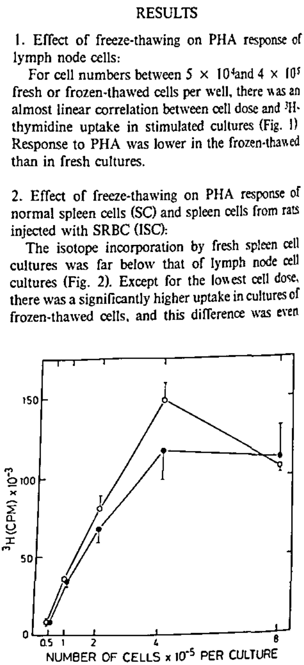


Fig 1. Dose response curves of cell proliferation by fresh (–O–) and frozen-thawed (–●–) lymph node cells stimulated with PHA. Vertical bars on mean values represent ± 1 standard deviation of triplicate cultures

macrophages to stimulated cultures of non-adherent spleen lymphocytes (Fig. 6). *Folch & Waksman* have previously described suppressor cell activity in spleen of normal rats (8, 9), and *Bach & Waksman* showed that this activity increased after injection of ovalbumin or high doses of SRBC (3, 4). This suppressor cell was glass wool adherent and was absent in carefully prepared »B rats« (3, 4, 8, 9). Thus, a lymphocyte was regarded as the suppressor cell. However, in later studies carragheenan reduced the suppressor activity in the cultures, thus pointing to a role for macrophages (4). Recent studies have demonstrated that suppressor macrophages in rat spleens inhibit lymphoproliferative response *in vitro* (15, 16, 19, 20), and this is more in line with the present observations. Suppressor macrophages are also demonstrated in peritoneal washings, and the suppressive effects are qualitatively the same, but probably stronger (2, 14, 15, 19).

It should be noted, however, that macrophages do not exclusively act as inhibitors. When small numbers of macrophages were added to cultures of macrophage-depleted spleen cells, there was a positive effect on ^{3}H-thymidine uptake in stimulated cultures (Fig. 6). Whether or not macrophages are necessary for mitogen stimulation remains controversial (21), but they are known to have a positive effect on lymphocyte viability *in vitro* (6, 18). From the present experiments and from those of *Folch & Waksman* (7), it is evident that inhibition of ^{3}H-thymidine incorporation of stimulated cultures preferentially occurred at higher cell concentrations. This may indicate that there must be a threshold number of suppressor cells to be effective, or that suppressor cells exert a direct cell-to-cell effect.

The aim of this study was also to explain the unexpected increase in thymidine incorporation by frozen-thawed spleen cell cultures. In normal spleen cell suspensions, this difference was small but significant at cell doses of 2×10^5 cells per well or above, and clearly the opposite to that of lymph node lymphocytes. By injection of heavy doses of SRBC 24 h before sacrifice (3), a procedure which probably activated the macrophages, the increased isotope incorporation by stimulated spleen cells after freeze-thawing could be made greater, and this model was therefore chosen for further experiments. Depletion or intoxication of macrophages seemed to mimic the effects of freeze-thawing, or vice versa. Furthermore, when equal numbers of fresh and frozen-thawed macrophages were added to stimulated cultures of non-adherent spleen cells, inhibition was greater by fresh macrophages. Macrophages are more sensitive to freeze-thaw injury than lymphocytes (10, 13, 23). Thus, freezing of a suspension consisting of both lymphocytes and macrophages will give an enrichment of lymphocytes with a correspondingly lower percentage of macrophages, as shown by the esterase staining of the frozen-thawed suspensions. Freeze-thawing of spleen cells will cause damage to both lymphocytes (as shown by the effects of freezing on purified spleen lymphocytes) and macrophages. The effect on lymphocytes will give a lower response of frozen-thawed cultures, but this is more than counterbalanced by the greater loss of inhibitory macrophages. Evidence for this theory is also given by the experiment where non-adherent spleen cells were mixed with 10% macrophages, and cultures of frozen-thawed cells had a higher response to PHA than fresh cells.

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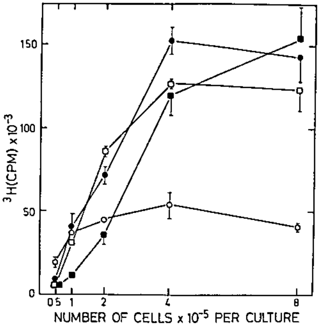


Fig 4. Dose response curves of cell proliferation by PHA-stimulated spleen cells from rats injected i v. with SRBC. (–O–) fresh spleen cells, (–●–) fresh spleen cells cultured with 1 mg/ml carragheenan, (–□–) frozen-thawed spleen cells, and (–■–) frozen-thawed spleen cells cultured with 1 mg/ml carragheenan Vertical bars on mean values represent ± 1 standard deviation of triplicate cultures

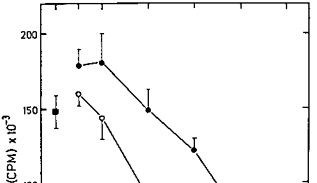


Fig 5 Response to PHA by 2 × 10^5 non-adherent spleen cells cultured alone (■) and with increasing concentrations of fresh (–O–) or frozen-thawed (–●–) macrophages Vertical bars on mean values represent ± 1 standard deviation of triplicate cultures.

TABLE 1. *Effect of Freeze-thawing on PHA Stimulation of Mixed (1:10) Macrophage: Column Purified Spleen Cell Cultures*

Exp No.	^{3}H-thymidine incorporation (cpm) Fresh cells	Frozen-thawed cells
1	25 729 ± 4 178	55 986 ± 4 930
2	33 168 ± 1 782	71 817 ± 4 685
3	16 741 ± 1 375	54 166 ± 947
4	35 622 ± 4 611	58 741 ± 3 735

2 × 10^5 cells of a mixed 1:10 peritoneal macrophage glass wool xolumn purified spleen cell suspension were cultured with PHA, either fresh or after freeze-thawing of the mixture.

6. Effect of freeze-thawing on the PHA response of a mixture of non-adherent spleen cells and peritoneal macrophages:

The following experiment was designed as a model for freezing whole spleen cell suspensions containing both macrophage-like suppressor cells and lymphocytes. NASC were mixed with 10% peritoneal macrophages and fresh and frozen-thawed cells from this suspension were cultured at a concentration of 2 × 10^5 cells per well and stimulated with PHA. Cultures of frozen-thawed cells incorporated 1.6 to 3.2 times more labelled thymidine than their fresh counterparts (Table 1) Freeze-thawing of this suspension reduced the numbers of esterase positive cells from 7–10% of fresh cells to 3–5% after post-thaw washing

DISCUSSION

It is now well documented that the low proliferative response in rat spleen cell cultures is due to the activity of suppressor cells (4, 7, 8, 9, 15, 16, 17, 19, 20, 24). Some controversy exists regarding the nature of these cells. The present experiments in which ISC were depleted of adherent or phagocytic cells showed an increased incorporation of labelled thymidine in stimulated cultures as compared to whole spleen cultures. Likewise, intoxication of macrophages by carragheenan in the cultures elevated the isotope uptake. Neutralizing the splenic macrophages by these procedures made the response of spleen cells to stimulation with PHA more like that of lymph node cells. Based on these findings, it is difficult to offer an explanation which does not point to macrophages as suppressor cells. This hypothesis was further supported when suppression could be restored by adding peritoneal

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STIMULATION OF HUMAN FETAL LYMPHOCYTES BY LIPOPOLYSACCHARIDE B IN CULTURE. STUDIES ON CELLS CIRCULATING IN MATERNAL BLOOD

S. BJORKBOM, O TURUNEN, C LUNDQVIST and A DE LA CHAPELLE

Department of Medical Genetics, University of Helsinki, and Folkhalsan Institute of Genetics, Helsinki, Finland

Bjorkbom, S, Turunen, O, Lundqvist, C & de la Chapelle, A Stimulation of human fetal lymphocytes by lipopolysaccharide B in culture Studies on cells circulating in maternal blood Acta path microbiol scand Sect C, 86 159–164, 1978

Lipopolysaccharide B (LPS) was used in attempts to stimulate fetal cells circulating in the maternal blood during pregnancy Crystalline leukoagglutinin (LA), the mitogenic properties of which are identical with those of phytohemagglutinin, was used as a reference mitogen When artificial mixtures of varying proportions of lymphocytes from mothers (XX) and their newborn male infants (XY) were co-cultured in the presence of these mitogens, LPS brought about a definite enrichment of XY mitoses, indicating that even under these conditions of co-culture, LPS preferentially, though not exclusively, stimulates the infant cells When cells from the blood of 13 women pregnant in the second trimester with a male fetus were cultured with LPS as a mitogen, all mitoses were found to be XX. Since interphase Y chromatin occurred in uncultured lymphocytes from these women (cells containing Y chromatin were found in 5 out of 6 women tested), we conclude that LPS is unable to stimulate, or at least induce mitoses, in fetal cells circulating in the blood of pregnant women. The nature of these cells is discussed

Key words Fetal lymphocytes, maternal blood, mitogens, lipopolysaccharide B.

O Turunen, Department of Medical Genetics, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland

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It is a well-known fact that cells interpreted as being of fetal origin occur in the blood of most pregnant women (5, 6, 11, 13, 14, 17, 18) If these cells could be isolated or purified they might provide a useful tool for prenatal diagnostic determinations of fetal genetic diseases. If these fetal cells circulating in the mother's blood could be selectively induced to transform and divide by Lipopolysaccharide B (LPS) then they might also be isolated and purified (16). The aim of the present work was to test the effect of LPS and other mitogens on cells from pregnant women, in an attempt to obtain fetal mitoses

MATERIAL AND METHODS

Cell sources Citrate blood samples were obtained from healthy mothers within 12 h of delivery, umbilical cords at delivery and women pregnant in the second trimester with a male fetus (karyotype determined later by amniocentesis)

Isolation and culture of cells When whole blood was not used, the lymphocytes were separated by the Isopaque-Ficoll method (4) and washed twice in MEM. Whole blood or mononuclear cells were cultured on disposable polystyrene microplates (Cooke Microtiter® System, Sterilin Ltd, Teddington, Middlesex TW 11 8QZ, UK). Each well contained 1 to 3 × 10^5 mononuclear cells in a total of 80–90% RPMI 1640 (Gibco Bio-Cult, Glasgow, Scotland) and 10–20% heat-

inactivated pooled human AB serum. L-glutamine (1.28 mM), penicillin (32 IU/ml) and Streptomycin (0.032 µg/ml) were added. The incubation was carried out at 37° C in an atmosphere of air containing 5% CO_2. The proportions of maternal and cord blood lymphocytes in the artificial mixtures were 99.1, 90:10 and 50.50.

Mitogens. Crystallized leukoagglutinin (LA) from Pharmacia Fine Chemicals, Sweden, was used as a reference mitogen throughout this study, the final concentration being 20 µg per ml of culture medium. Lipopolysaccharide B (LPS) from *Escherichia coli* 055.B5 was purchased from Difco Laboratories and boiled in a water bath for 2 h before use in a final concentration of 40 µg/ml.

Mitotic activity. Two hours before harvest 0 05 µg of desacetylmethylcolchicine (Colcemid®, Ciba, Basel Switzerland) was added to each well. At harvest 4–5 ml 0.075 M KCl (hypotonic) was added to the contents of 3 to 4 wells. After 10 minutes the suspension was centrifuged, the supernatant discarded and the cell pellet fixed in a 3:1 mixture of methanol and acetic acid After fixation for at least 0.5 h, slides were prepared by the airdrying method. They were then stained with Acranil® dihydrochloride, Pt 155735, Bayer Leverkusen, Germany.

RESULTS

Leukoagglutinin as a reference mitogen. LPS, which is a polyclonal B-cell activator, is a weak mitogen for human peripheral blood lymphocytes (9). When testing its mitogenic activity, comparison should be made with the effect of a known strong mitogen. For this purpose we chose leukoagglutinin (LA), the pure crystalline form of phytohemagglutinin (PHA). Leukoagglutinin (LA) lacks erythroagglutinating and other undesirable qualities (10).

The effect of LPS in artificial mixtures of fetal and maternal lymphocytes. The proportion of fetal cells circulating in maternal blood is of the order of 1 fetal cell, or less, per 1000 maternal cells (11, 18) Hence there is an overwhelming majority of maternal cells. To test whether LPS selectively stimulates fetal cells appearing in the presence of adult cells, we prepared artificial mixtures of known numbers of cells from male cord blood lymphocytes obtained at delivery, and lymphocytes from the mother. We have previously shown that if such cells are cultured separately, cord cells are stimulated with LPS, whereas maternal cells are definitely not stimulated (16). The outcome of the experiments using mixtures of cells is summarized in Figs. 1 and 2. If the proportion of input fetal (XY) cells is 50% (Fig. 1c), then 45–82% of all mitoses produced are XY, irrespective of whether the mitogen is LA or LPS, or if no mitogen at all is added. By contrast, if the input proportion of XY cells was 1 per cent (Fig. 1a), LA produced 1–5% XY mitoses, while LPS produced 22–45%. Extremely few mitoses, mostly XX, arose in cultures to which no mitogen had been added (Fig. 1a). When the proportion of XY cells in the mixture was 10% the proportions of the two types of mitoses (Fig. 1b) were intermediate between those already described.

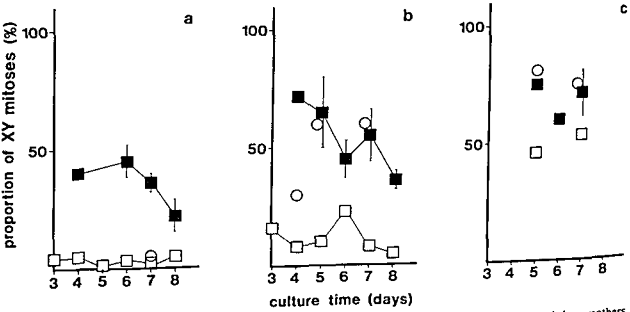


Fig. 1. Proportion of XY mitoses in cultures of artificially mixed cells from male (XY) newborns and their mothers (XX), using different mitogens. Lymphocyte cultures with 1 5 × 10^6 cells per ml (LPS and controls without mitogen) and 5 × 10^5 cells per ml (LA) were used. Mitoses were arrested by treatment with Colcemid for the last two hours of culture. Proportion of XY cells at the beginning of culture a) 1%, b) 10%, c) 50%.
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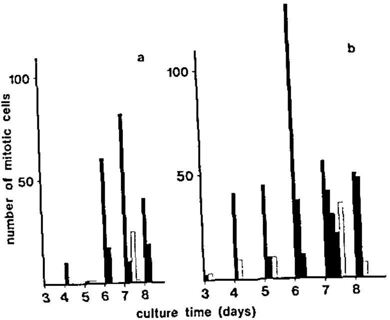


Fig 2 Number of Colcemid-arrested mitoses in cultures of artificially mixed lymphocytes from newborn males (XY) and their mothers (XX) Summary of four experiments Cultures with 1 5 × 10^6 lymphocytes per ml were used Proportion of XY cells at the beginning of culture a) 1%, b) 10% White columns no mitogen, black columns. LPS

To evaluate these results fully, the total number of mitoses in each type of mixture must be known. Figs 2a and b show the numbers of mitoses from the 1% and 10% mixtures, respectively (when the proportions of the two cell types were equal (50%) large numbers of mitoses occurred). Taken together, these experiments indicate that the mixed leukocyte reaction (no mitogen used) provides, not unexpectedly, many mitoses under 10 90 and 50.50 mixture conditions, while with the proportion 1 99, only few mitoses occur On the other hand, LPS produces numerous mitoses even in the 1 99 mixture, and the proportion of XY (fetal) mitoses is greatly enriched with respect to the input. The same trend is obvious in the 10.90 mixture Under all three mixture conditions, the proportion of mitoses produced by LA does not differ greatly from the input proportion. One further point should be noted It is obvious that the stimulation of maternal cells by LPS is enhanced by the mixed leukocyte reaction, since so many XX mitoses are produced with LPS e g in the 1 99 mixture, although we have previously shown that LPS alone is unable to stimulate adult human lymphocytes (16).

We conclude from these studies that cord cells co-cultured with adult cells are preferentially, though not exclusively, stimulated by LPS in vitro

The effect of LPS on cells from the blood of pregnant women If LPS does selectively stimulate those fetal cells that occur in the blood of pregnant women, then culture of cells with LPS from women pregnant with a male fetus should produce at least some XY mitoses.

A preliminary series of experiments was designed to test, by the interphase Y chromatin (Y body) technique, whether Y chromatin-containing lymphocytes actually do occur in women expecting a boy, but not in women expecting a girl Blood was obtained from 13 women attending a prenatal diagnostic service. Slides of uncultured leukocytes were prepared for quinacrine fluorescence analysis of interphase Y chromatin, which was performed before the sex of the fetus was known. To avoid misinterpretations of unusually large brightly fluorescent autosomal regions (11, 13), mitoses from all 13 women were first screened in the fluorescence microscope. Two cases had to be excluded from further analysis because of bright autosomal fluorescence After the samples had been taken from the mothers, amniocentesis was performed for various reasons, mostly advanced maternal age. Alternatively, induced abortion was performed for different reasons. Thus the sex of the child became known either through the karyotype or phenotype, or both. The results from the 11 mothers tested are shown in Table 1. As can be seen, of the 6 women pregnant with a male fetus, 5 had at least 1 cell out of 5000 with Y chromatin. None of the 5 women with a

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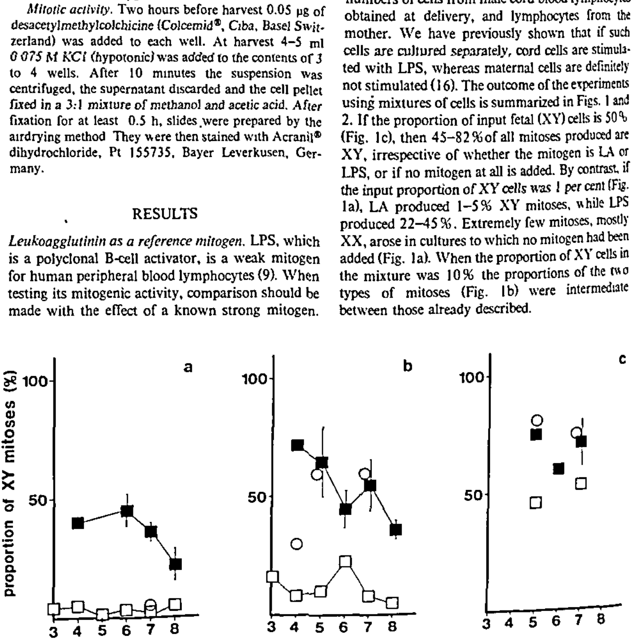


Fig. 1. Proportion of XY mitoses in cultures of artificially mixed cells from male (XY) newborns and their mothers (XX), *using different mitogens.* Lymphocyte cultures with 1 5 × 10^6 cells per ml (LPS and controls without mitogen) and 5 × 10^5 cells per ml (LA) were used. Mitoses were arrested by treatment with Colcemid for the *last two hours* of culture. Proportion of XY cells at the beginning of culture a) 1%, b) 10%, c) 50%.
Symbols: ■ = LPS, □ = LA; ○ = no mitogen.

spread is unambiguous with quinacrine fluorescence Blood obtained from 13 pregnant women attending a prenatal diagnostic service was used after the sex of the fetus was determined to be male as indicated above. Isopaque-Ficoll isolated lymphocytes and/or whole blood cells were cultured for various periods of time without mitogen and with LPS Mitoses were arrested by treatment with Colcemid for the last two hours of culture. Slides were prepared using ordinary cytogenetic techniques to obtain good mitotic spreads. Mitoses were localized by phase contrast microscopy of unstained slides The slides were then stained with quinacrine, the mitoses relocalized and studied in the fluorescence microscope for the presence of a Y chromosome The results from the 13 patients are shown in Table 2 The first six patients are identical with the first six in Table 1 There were only a few mitoses in one patient when no mitogen was used All were XX LPS produced varying proportions of mitoses in 6 of the 13 patients, but even here not a single XY mitosis was seen Since we know that Y chromatin-positive interphase cells occurred in at least 5 of these women we conclude that mitoses produced by LPS under these conditions are maternal and hence LPS is unable to stimulate, or at least bring about division, in fetal cells circulating in the maternal blood during pregnancy

DISCUSSION

The present results are in good agreement with our earlier observations (16) and show that it is possible to enrich umbilical cord lymphocytes in artificial mixtures with maternal lymphocytes It should be kept in mind, however, that umbilical cord lymphocytes may differ from fetal lymphocytes in responsiveness to various mitogens On the other hand, fetal and adult lymphocytes do not differ greatly e g. in their proliferation response against xenogeneic and allogeneic lymphocytes (2)

When artificially mixed lymphocyte cultures are studied, the interaction between maternal and cord blood lymphocytes has to be considered It has been shown (7) that a 50 50 mixture of viable maternal and neonatal lymphocytes yields more than 50% XY (fetal) metaphases because of inhibition of the divisions of maternal cells In most mixed leukocyte studies, however, the two cell types were mixed in equal proportions, while in our experiments we also tested mixtures in which the percentage of cord blood lymphocytes was 1 and 10 Under such circumstances the suppressor activity of the neonatal lymphocytes may be greatly reduced When mixed lymphocyte cultures were stimulated by mitogens the suppression of mitoses of maternal cells did not seem to be altered (8)

It has been estimated that the proportion of fetal cells in a pregnant woman is about 1 fetal cell per 1000–5000 maternal cells (11, 18). The mixture containing 1% cord blood lymphocytes and 99% maternal lymphocytes was designed to resemble the conditions in the peripheral blood of pregnant women In this experiment a 45-fold enrichment of XY (fetal) mitoses was achieved when LPS was used as mitogen. The maximum stimulation occurred on day 6 of culture, which is usual for LPS (16) LA induced a somewhat higher proportion of XY mitoses (5%) than would be expected (1%). This could be due to a greater sensitivity of cord blood lymphocytes to stimulation by LA, compared with maternal lymphocytes, but the mixed lymphocyte reaction may also be responsible for this, at least in part In this context it should be noted that the stimulatory mechanism known as the mixed leukocyte reaction mostly results in fewer mitoses than in cultures containing soluble stimulants (1) and is especially weak in mixtures between relatives (3). Since we always co-cultured cells from mothers and their newborn children, the mixed leukocyte reaction should have been of limited magnitude.

In the second part of this study LPS was used to stimulate cultures of lymphocytes from women pregnant in the second trimester with a male fetus Y-bodies occurred in the interphase nuclei of some of these lymphocytes, indicating the presence of fetal cells However, not a single XY mitosis could be detected amongst the metaphases of the mothers when lymphocytes or whole blood were cultured in the presence of LPS There were, however, some XX (maternal) mitoses in some cultures Even in cultures without any mitogen, maternal mitoses occurred Thus most of the maternal mitoses in the cultures with LPS can be assumed to have arisen spontaneously.

Our series consisting of 13 mothers pregnant with a male fetus is small, but at least a few detectable XY (fetal) mitoses in some of the cases were expected, since it is believed that the fetal cells occurring in the maternal circulation are lymphocytes or lymphocyte-like cells. They could, however, be something else, e g monocytes (12) and become eliminated during the Isopaque-Ficoll isolation procedure. This point was tested by using whole blood cultures from 6 women, but even so no XY mitoses were found. If the fetal cells are lymphocytes then they must be unresponsive to LPS under these culture conditions. They might be masked (15). Thus not even LPS, which *stimulates fetal and* cord blood lymphocytes in separate cultures, is unable to activate those fetal cells that circulate in

TABLE 1. *Search for Y Chromatin-Positive Cells in 11 Pregnant Women in the Second Trimester. The Determinations were Made Before the Sex of the Fetus Became Known*

Initials and age (years) of mother	No. and sex of previous children; abortions	No. of Y-bodies per 5000 interphase cells	Sex of fetus
M.L. 40	–	27	M
K.N. 39	–	0	M
A.E 40	2 F; 1 A (1959)	2	M
E.S. 40	1 M (1962)	1	M
P.P. 15	–	1	M
A.Y. 23	1 F	2	M
S.V. 42	–	0	F
M.A. 39	–	0	F
T.H 28	2 F	0	F
E.N. 40	–	0	F
R.H. 39	–	0	F

M = male
F = female
A = spontaneous abortion

female fetus had any Y chromatin-positive cells. Hence our results were similar to those obtained in previous studies (11, 13, 18) and indicated that fetal cells containing Y chromatin do occur in the blood of most women pregnant with a male fetus.

The search for XY mitoses in LPS-stimulated cultures from pregnant women was then continued in females actually known to expect a boy. Blind testing was no longer judged to be necessary since the identification of a Y chromosome in a mitotic

TABLE 2 *Search for XX and XY Mitoses in Cultures of Peripheral Blood Cells from 13 Women Pregnant with a Male Fetus in the Second Trimester (14–17 Weeks) Only XX Mitoses were Found*

Initials and age (years) of mothers		Week of pregnancy	No and sex of previous children, abortions	Days of culture	No of mitoses[a] per 10^5 cells[b]		
					Without mitogen	*LPS*	
					Lymphocytes[c]	Whole blood	Lymphocytes[c]
M.L	40	16	–	4,5,6,7	N D	N D	0
K N	39	15	–	3,4,7,8	N D	0.05	0.08
A E	40	15	2 F 1 A (1959)	4,5,6,7	N D	N D	0
E.S	40	15	1 M (1962)	3,6,7,8	N D	0	0
P P	15	15	–	5,6,7,8	N D	0	0
A Y	23	16	1 F	3,6,7	0	0	0.04
A R.	29	16	1 F	5	N D	N D	0.25
V T	40	17	–	6,7	0	N D	1.16
S.P	25	15	1 F	4,6	0.91	N D	0.16
M M	36	16	–	4,6,7	0	N D	0.62
M T	40	14	1 A (1967)	6,7	N D	0	0
S.H	19	16	–	6,7,8	0	N D	0
P T	17	16	–	3,6,7	0	0	0

N.D. = not determined
M = male
F = female
A = spontaneous abortion (sex of fetus unknown)

a) All mitoses were XX
b) Number of mitoses observed divided by the number of cells present at the start of culture
c) Cells separated by the I-F method (cf. text)

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INFLUENCE OF PHENYLBUTAZONE ON LEUKOCYTE CHEMILUMINESCENCE AND FUNCTION

CLAUS O SOLBERG, CRAIG D ALLRED and HARRY R. HILL

Division of Clinical Immunology, Department of Pediatrics, and the Department of Pathology, University of Utah Medical Center, Salt Lake City, UTAH 84132, U S A

Solberg, C. O, Allred, C D & Hill, H R: Influence of phenylbutazone on leukocyte chemiluminescence and function Acta path microbiol. scand Sect C, *86* 165–171, 1978.

The effect of phenylbutazone on human leukocyte chemiluminescence, phagocytosis and intracellular killing of bacteria has been examined A marked reduction of chemiluminescence and intracellular killing of bacteria was observed The effect on phagocytosis was less pronounced High drug concentrations nearly abolished light emission, and concentrations equivalent to those obtained in plasma during therapy caused a 25–30% reduction The effect occurred within less than 10 minutes No permanent effect upon resting cells was observed. Phenylbutazone reduced the effect of sodium azide on leukocyte chemiluminescence, indicating that the drug might also inhibit myeloperoxidase dependent chemiluminescence Whether these impairments of leukocyte function also take place *in vivo* resulting in enhanced susceptibility to infection remains unknown.

Key words phenylbutazone, leukocyte chemiluminescence, leukocyte function

C O Solberg, Medical Department B, N-5016 Haukeland sykehus, Norway

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Several disease syndromes characterized by recurrent infections have been related to defects in phagocytosis or intracellular killing of microorganisms by polymorphonuclear leukocytes (PMNs). Evidence is now accumulating that commonly used therapeutic agents might also influence granulocyte functions (5, 7, 14). In previous studies, it was found that phenylbutazone, a clinically useful anti-inflammatory drug, reduces rabbit (20) and human (12, 19) leukocyte function, the latter even at therapeutic concentrations Recently, a sensitive technique has been developed which measures granulocyte activation during phagocytosis (1) Following particle ingestion, the microbicidal mechanism of the granulocyte is activated and excited molecular oxygen and carbonyl groups are generated (1, 2) Upon relaxation to the ground state, these molecules emit photons resulting in a chemiluminescence, which can be detected and quantitated in a liquid scintillation counter Using this technique, we have compared the effect of phenylbutazone on leukocyte chemiluminescence, phagocytosis and intracellular killing of bacteria.

MATERIALS AND METHODS

Leukocytes

Heparinized blood (10 u/ml) from healthy human donors was allowed to settle for 30 to 60 minutes at 37° C The leukocyte rich plasma was removed, the cells sedimented and washed twice in sterile phosphate buffered saline (PBS = 4500 ml distilled water, 5 2 g Na_2HPO_4, 0 9 g KCL, 0 9 g KH_2PO_4, 36 g NaCL) by centrifugation at *100 g* for 10 minutes and resuspended in PBS to a concentration of $1\ 0 \times 10^7$ PMNs/ml

Opsonization of Zymosan

Zymosan particles (100 mg) were washed once in 10 ml PBS The pellet was then resuspended in normal

the maternal blood. This finding should be related to the fact that the cells in question are also unresponsive to leukoagglutinin (18) and a number of other mitogens (12).

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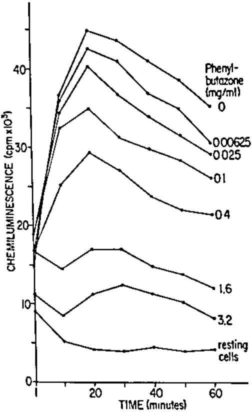


Fig 1 Effect of phenylbutazone on zymosan induced leukocyte chemiluminescence

valent to those obtained in plasma during therapy ie 50 – 120 μg/ml caused a 20–30% reduction, and even 6 25 μg/ml had a significant influence (Fig 1). Control experiments with leukocytes and phenylbutazone in concentrations from 0 00625 to 1 6 mg/ml demonstrated that 97 to 99% of the neutrophils resisted staining with trypan blue, indicating that their functional integrity was intact. However, when the leukocytes were incubated in the presence of 3.2 mg phenylbutazone/ml, only 75% of the neutrophils resisted staining with trypan blue

Pulse experiments with phenylbutazone were carried out to see how fast the drug might influence granulocyte function Phenylbutazone was added to the reaction mixture 10 and 20 minutes after initiation of the test procedure. A significant reduction in chemiluminescence occurred after 10 minutes (Fig 2) No reduction was observed in control tests pulsed with PBS

To study whether phenylbutazone was bound to the PMNs, leukocyte suspensions with 1.6 mg phenylbutazone per ml and controls containing no drug were incubated for 15 minutes at 37° C. Following incubation the cells were washed twice in PBS and exposed to opsonized zymosan as previously described. There was no residual effect of phenylbutazone upon subsequent exposure of the PMNs to zymosan.

In order to determine if phenylbutazone inhibits the chemiluminescence dependent upon myeloperoxidase (17), sodium azide, a myeloperoxidase inhibitor, was added to reaction mixtures with and without phenylbutazone (Fig. 3) In the absence of phenylbutazone, 65 mg/ml (1mM) sodium azide caused a 30% reduction of zymosan induced chemiluminescence. When low concentrations of phenylbutazone were utilized i.e. 0.1 mg/ml or less, sodium azide further reduced chemiluminescence by approximately 30%. In the presence of high concentrations of the drug i.e. 1.6 – 3.2 mg/ml, sodium azide had less effect, however, indicating that myeloperoxidase dependent chemiluminescence can be inhibited by high concentrations of phenylbutazone

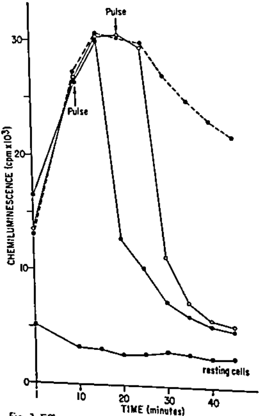


Fig 2. Effect on zymosan induced leukocyte chemiluminescence of 1 6 mg phenylbutazone/ml added to the reaction mixture at 10 and 20 minutes

human serum to a concentration of 10mg/ml. The suspension was mixed on a vortex mixer, capped and rotated on a roto rack (Fisher Scientific, Santa Clara, Calif.) for 60 min at 37° C. The opsonized particles were recovered by centrifugation at 1800 *g* for 5 min, the supernatant discarded and the particles resuspended in PBS to a final concentration of 10 mg/ml.

Drug

Sodium phenylbutazone (manufactured by Geigy, Basel, Switzerland) was dissolved in PBS and added to the incubation suspensions, giving final concentrations as indicated in the text and figures.

Reagent

Sodium azide (obtained from Sigma Chemical Co., St. Louis, MO) was dissolved in PBS and added to the incubation suspension, giving final concentrations as indicated in the text and figure.

Bacteria

^{3}H-labelled β-hemolytic streptococci, group B were prepared by incubating 0.1 ml of fresh bacterial suspension in Todd-Hewitt medium containing ^{3}H-leucine (5 μCi/ml) at 37° C for 24 hours. The concentration of bacteria was determined by streak plating serial dilutions of the suspension The bacteria were killed by heating at 60° C for 1 hour, washed 3 times in PBS, resuspended in PBS to 3.3×10^8/ml and stored in 5 ml aliquots at −20° C. Immediately prior to use the bacteria were thawed and opsonized by incubating a 6:1 (bacterial suspension· human serum) ratio for 30 mimutes at 37° C. The suspension was washed once by centrifugation and the bacteria resuspended to the original volume in medium 199

Staphylococcus aureus

»Oxford« (Heatley strain obtained from the National Collection of Type Cultures, Colindale, London 1958) was cultured overnight in Penassay broth (Difco), washed twice in 0 45% saline and suspended in PBS to an optical density of 0.6 at 620 nm in a Beckman spectrophotometer. This suspension was diluted in PBS to a concentration of $14-20 \times 10^7$ colony forming units/ml

Chemiluminescence Procedure

The chemiluminescence procedure has previously been described (3, 9, 10) Briefly, the control reaction mixture contained 2 8 ml PBS, 0 2 ml opsonized zymosan and 0.5 ml leukocyte suspension making a total volume of 3 5 ml The test reaction micture contained 2.7 ml PBS, 0 1 ml drug and/or reagent solution, 0 2 ml opsonized zymosan and 0.5 ml leukocyte suspension making a final volume of 3.5 ml The mixtures were made in Beckman Poly Q scintillation vials (Fullerton, Calif.) which were wrapped in aluminum foil and stored in dark for at least 24 hours prior to use The vial was immediately capped upon introduction of the reaction mixture and placed at ambient temperature in a Beckman LS-100$_c$ scintillation counter (Fullerton, Calif) out of phase and with one photomultiplier tube disconnected Duplicate vials for each test condition were counted at approximately 10 minute intervals for 60 minutes and the results recorded in mean counts per minute (CPM)

Phagocytosis Assay

Phagocytic uptake of radiolabelled bacteria by human PMNs was determined by a modification of the method previously described by *Mandell* (15) Monolayers of human leukocytes were prepared by incubating 0 3 ml leukocyte suspension on 18 × 18 mm glass coverslips at room temperature for 30 minutes. Nonadhering cells were washed off with PBS and 0.3 ml (1×10^8 organisms) of the opsonized, radiolabelled bacterial suspension was added to each coverslip Control suspensions contained only bacteria (1×10^8 organisms) in medium 199 while test suspensions also contained phenylbutazone. At intervals of 1, 10, 20, 30, 40, 50 minutes the coverslips were washed with PBS, placed in scintillation vials containing 5.0 ml aquasol (New England Nuclear, Boston, Mass.) and counted 5 minutes in a Beckman LS-100$_c$ scintillation counter Microscopic examination of control coverslips stained at each time interval showed the monolayers to be evenly distributed, and the number of adherent cells ($5 \times 10^5 - 1.5 \times 10^6$ per coverslip) was estimated by counting cells in 5 random grids at 400 power and multiplying the average by the number of grids (20227) per coverslip The accumulated CPM/10^6 cells were calculated at each interval and plotted against time Controls with bacterial suspensions added to coverslips without cell monolayers showed no accumulation of radioactivity.

Assay for Phagocytosis and Intracellular Killing of Bacteria

The phagocytic and bactericidal activities of the granulocytes were determined as previously described (18) 0.5 ml leukocyte suspension, 0 4 ml 25% pooled human serum as opsonin and 0 1 ml bacteria suspension were added to plastic tubes This provided about 3 bacteria per PMN and a final concentration of 10% serum. In the tests with phenylbutazone, the drug was added to the leukocyte suspension and diluted serum immediately before mixing with the bacteria suspension The tubes were incubated at 37° C and samples removed at prescribed intervals for the determinations of the total number of viable bacteria and the number of viable intracellular bacteria as previously described (18) The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the number of viable intracellular bacteria (18). The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (18)

Statistical Method

The Wilcoxon-test for 2 samples was used

RESULTS

Chemiluminescence

Phenylbutazone markedly reduced chemiluminescence. High drug concentration i e 3.2 mg/ml nearly blocked light emission; concentrations equi-

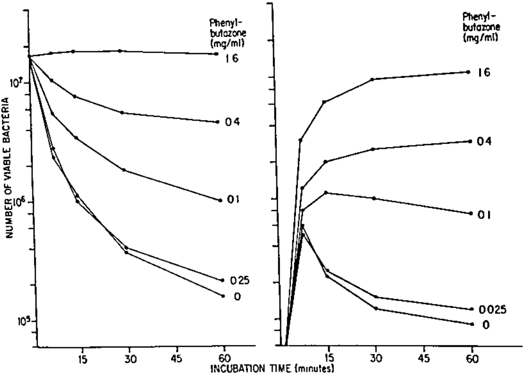


Fig 5 Total number of viable bacteria (left) and number of viable intracellular bacteria (right) during incubation of leukocyte-bacteria suspensions with and without phenylbutazone (mean of 5 experiments). The differences between 0 and 0 025 mg phenylbutazone/ml were not significant ($p \geqq 0\ 1$) The differences between the other groups at 7 5, 15, 30 and 60 minutes were significant ($p < 0\ 01$)

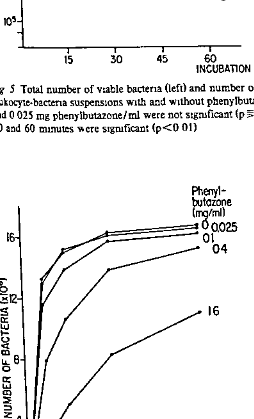


Fig 6 Number of phagocytized bacteria during incubation of leukocyte-bacteria suspensions with and without phenylbutazone (mean of 5 experiments) The differences between 0 and 0 025 mg phenylbutazone/ml were not significant ($p \geqq 0\ 1$) The differences between the other groups at 7 5, 15, 30 and 60 minutes were significant ($p < 0\ 01$)

DISCUSSION

Phagocytosis and intracellular killing of bacteria and fungi represent cornerstones of the host defence mechanisms. Whether these functions may be influenced by commonly used drugs such as phenylbutazone is, therefore, of significant clinical importance. In the present study, as well as in previous studies (12, 19, 20) phenylbutazone caused a marked reduction in the intracellular killing of bacteria by human granulocytes. The influence of the drug on zymosan induced chemiluminescence was also striking: 3.2 mg phenylbutazone/ml reduced the chemiluminescence by as much as 80–90% and even 6.25 µg/ml caused a significant reduction. However, there seemed to be no strict correlation between the reduction of chemilumine-

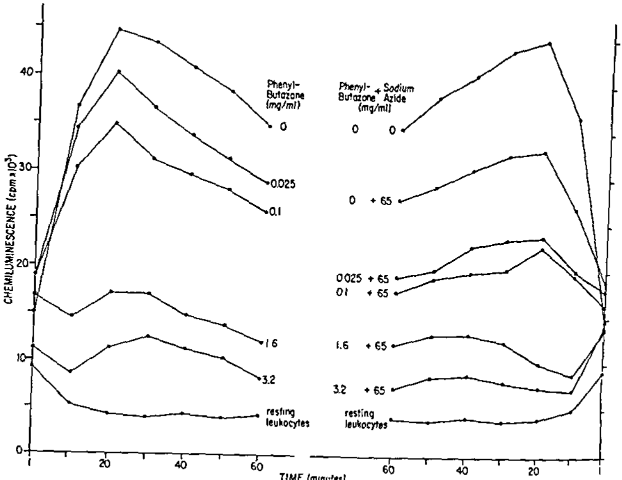


Fig. 3. Effect of phenylbutazone (left) *and sodium azide (1 mM)* plus phenylbutazone (right) on zymosan induced leukocyte chemiluminescence.

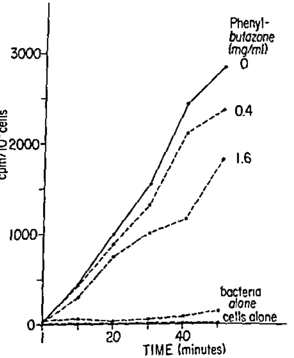


Fig 4 Effect of phenylbutazone on leukocyte phagocytosis of ^{3}H-labelled beta-hemolytic streptococci, *group B*

Phagocytosis and Intracellular Killing of Bacteria

In the tests with leukocyte monolayers and ^{3}H-labelled streptococci, a 40% reduction of normal *phagocytosis at 50 minutes was observed in the* presence of *1.6* mg phenylbutazone and a 15% reduction in the presence of *0.4* mg/ml (Fig. 4) Lower drug concentrations (0.1 and 0.025 mg/ml) did not significantly influence phagocytosis.

In the tests with leukocytes and *Staph. aureus*, a marked reduction of total colony counts was observed in the control tests (no phenylbutazone) and very few intracellular bacteria remained viable after incubation for 60 minutes (Fig. 5), demonstrating both rapid phagocytosis (Fig 6) and intracellular killing of bacteria. In the tests containing *1.6 mg* phenylbutazone/ml, however, nearly all bacteria remained viable after incubation for *60 minutes*, and large numbers were located *intracellularly*, demonstrating *markedly reduced intracellular killing of bacteria and 40–50% reduction of phagocytosis The influence of lower concentrations of* phenylbutazone *on phagocytosis and intracellular killing of Staph. aureus by* PMNs *was proportional* to the level of the drug, and 25 μg phenylbutazone/ ml *did not significantly influence* PMN functions.

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scence and bactericidal activity of the cells. The lowest drug concentration i.e. 6.25 μg/ml which significantly reduced the chemiluminescence, did not influence the intracellular killing of the bacteria and drug concentrations which seemed almost to abolish the bactericidal activity i.e. 1.6 mg/ml and in addition caused a 40–50 % reduction of phagocytosis (engulfment), caused only a 60 % reduction of light emission, indicating that the chemilumiscence does not only reflect the microbicidal capacity of the cells.

The reason for the marked inhibition by phenylbutazone of light emission by phagocytizing granulocytes is not obvious. However, the microbicidal action of leukocytes is supposed to proceed in part through oxygen dependent reactions. Following particle ingestion, molecular oxygen is reduced to superoxide radical anion which, together with its products, may react directly with the microorganism (4, 11), or hydrogen peroxide and a halide may act as substrates for the halogenation of bacteria by myeloperoxidase (13). Both superoxide – and myeloperoxidase – dependent reactions exhibit chemiluminescence *in vitro* and they appear to be the major sources of light emitted during phagocytosis (17). In previous studies, it has been demonstrated that phenylbutazone, mainly by acting on two key enzymes (G6PDH and 6PGDH) in the hexosemonophosphate shunt, inhibits the shunt activity, resulting in markedly reduced hydrogen peroxide production (20). The close linkage between hydrogen peroxide and superoxide anion generation indicates that superoxide production and superoxide dependent bactericidal activity are also reduced. This could be in agreement with the observation that emission of luminescence by phagocytizing cells correlates with the activity of the hexosemonophosphate shunt (1).

Whether phenylbutazone can also inhibit the bactericidal activity of myeloperoxidase is not well understood. However, hydrogen peroxide has been shown to be a lysosomal labilizing agent (21) Accordingly, inhibition by phenylbutazone of hydrogen peroxide production may prevent lysosomal degradation resulting in decreased availability of myeloperoxidase. Furthermore, since hydrogen peroxide together with myeloperoxidase and a halide act synergistically as a potent bactericidal system, lack of hydrogen peroxide will inhibit the activity of this system and probably also the myeloperoxidase dependent chemiluminescence The finding in our study that high concentrations of phenylbutazone markedly reduce the depression of chemiluminescence associated with sodium azide, suggests that this agent also acts to inhibit myeloperoxidase dependent chemiluminescence.

Whether the inhibition of leukocyte function demonstrated in this study also takes place *in vivo* reducing antimicrobial host defence, is not known However, following oral administration of therapeutic doses of phenylbutazone, plasma levels of 50 to 120 μg/ml are usually reached (6, 8). The biotransformation of the drug is also slow, averaging about 20 % per day and plasma levels of 20 to 40 μg/ml may be found as long as 7 days after therapy is discontinued (8). In the present study, 100 μg/ml caused a marked reduction in chemiluminescence and intracellular killing of bacteria by the granulocytes and even 6.25 μg/ml reduced the chemiluminescence. Finally, as demonstrated in the present study, the *in vitro* influence of the drug on granulocyte function is also rapid. However, the protein binding is high, and this may reduce the *in vivo* effect of the drug. Accordingly, to evaluate the clinical relevance of our findings, extensive clinical studies may be indicated.

The reason for the therapeutic effect of phenylbutazone in non-infectious arthritis inflammation remains speculative. However, typical for these conditions are the depolymerization of the hyaluronic acid and the degradation of the synovial fluid which may be caused by the hydroxyl radical produced by enzymatically generated superoxide radical reacting with hydrogen peroxide (16) The most likely physiological source of superoxide radical appears to be metabolically activated granulocytes which appear in large numbers in the inflamed joints and release superoxide not only into the phagocytic vacuole but also into the surrounding tissue. Accordingly, the therapeutic effect of phenylbutazone in arthritis inflammation may be due to inhibition of superoxide radical and hydrogen peroxide production by the granulocytes. This is also in agreement with the findings that superoxide dismutase and catalase protect synovial fluid against such degradation *in vitro* (16).

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LOCALIZATION OF Fc RECEPTORS IN HUMAN AND RAT MALIGNANT TISSUES

OLAV TÖNDER, ENGIKOLAI C. KRISHNAN, PAUL A. MORSE, JR., WILLIAM R. JEWELL, and LOREN J HUMPHREY

Department of Surgery, University of Kansas Medical Center, Kansas City, Kansas, USA; and Broegelmann Research Laboratory for Microbiology, School of Medicine, University of Bergen, Bergen, Norway

Tönder, O, Krishnan, E. C, Morse, P A, Jr., Jewell, W. R & Humphrey, L. J. Localization of Fc receptors in human and rat malignant tissues Acta path microbiol scand Sect. C, 86 173–178, 1978

Three human malignant tumors and a rat carcinoma (Walker 256), which all showed strong Fc receptor activity in tests with sheep erythrocytes (E) sensitized with rabbit IgG antibodies (A), were used to study the distribution of the receptor in subcellular fractions isolated by differential centrifugation Smears of the three crude sediments obtained (nuclei, mitochondria rich and membrane rich) all possessed receptor activity EA incubated in the suspension of cell fractions did not attach to Fc receptor positive tissue sections or to cells in suspension Cells from which the surface receptors had been eluted still showed activity in sections of cell pellets Apparently the Fc activity is connected with both external and internal membranes

Key words Fc receptor, tumor-associated, localization.

Olav Tonder, Broegelmann Research Laboratory for Microbiology, N-5016 Haukeland sykehus, Bergen, Norway

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Several investigations have shown that human and animal malignant tissues possess receptors for Fcγ (for refs. see 3, 4) The receptors have special affinity for Fc appearing in antigen-antibody complexes, and can be detected using either sections of frozen, unfixed tumors which adsorb sheep erythrocytes (E) sensitized with rabbit IgG antibodies (A), or using cells in suspension forming rosettes with EA However, the reactivity was usually weaker with cells in suspension than with tissue sections (8) This apparent discrepancy might depend not only upon the higher sensitivity of the tissue section technique, but also on the localization of receptors within the cells in addition to the localization on the surface The surface receptors may also be covered with IgG *in vivo*, leaving mainly internal receptors available for *in vitro* testing with tissue sections.

By screening some mouse and rat tumors for Fc receptors, we observed that a rat carcinoma (Walker tumor 256) gave strong reactions using tissue sections, and a high percentage of single cells gave complete rosettes with EA. Since this tumor could be supplied in large quantities we decided to use it for model studies on the cellular localization of the Fc receptor. Comparison with human tumors was performed according to availability of material. The results are presented herein.

MATERIALS AND METHODS

Tissues

Specimens from human malignant tumors were obtained from tissue of organs removed during surgery. Histological investigations and classification of tumors was routinely performed in the Department of Pathology (Kansas City or Bergen). Only tumors which showed an activity of two or higher in the Fc test with tissue sections (see below) were selected.

Rat tumor Walker 256 (carcinosarcoma) was originally obtained from Dr. *Schloerb*, Department of Surgery, Kansas City, and was maintained by transplantation s.c. to Sprague Dawley rats every fourth week

TABLE 1. *Fc Receptors in Sections of Malignant Tumors Activity of Various Tumors in Adsorption of EA Sensitized by Various Amounts of IgG Antibodies Distribution of Numbers of Tissue Types According to Degree of Sensitization Giving End Point Reactions*

Type of tissue	No	EA with no of agglutinating units of A				
		$^1/_2$	$^1/_4$	$^1/_8$	$^1/_{16}$	$^1/_{32}$
Human tumors						
Melanoma	3	1	1	1		
Leiomyosarcoma	1					1
Carcinoma of						
Lung	2			1	1	
Stomach	2			1	1	
Bladder	1			1		
Colon	1	1				
Breast	5	3	1	1		
at tumor						
Walker 256	(10 individual)				6	4

rom the lack of reactivity with EA made with ʽ(ab')₂fragments of the sensitizing antibody. Like-vise there was no reaction with sections left in PBS it 56° C for 15 min, while neuraminidase treatment of the sections increased the reactivity. These characteristics are similar to those of human malignant tumors (10), and we therefore considered the murine tumor a good experimental model Most of the following experiments were primarily performed with the Walker tumor since sufficient amounts were readily available, and the human tumors were employed in final tests for comparison, and confirmation or control of the findings with the rat tumor

The cell suspensions were tested for percentage of rosette forming cells using E sensitized with various amounts of A as described This test was dependent on having good dispersion of the tissue cells, in addition to the rat tumor, only one melanoma, one breast carcinoma and one bladder carcinoma gave satisfactory preparations Representative results of these tests are shown in Fig 1

As described earlier (8, 10) the rosette formation is dependent on the degree of sensitization of the EA, but apparently there was no increase in numbers of rosettes by increasing the amount of IgG above 1 agglutinating unit. However, counting rosettes with these strongly sensitized EA was hampered by the spontaneous clumping of the sensitized EA Experiments to overcome this difficulty were performed using formalinized E which form only small clumps in excess of antibodies The percentage of rosettes obtained were parallel to the numbers obtained with untreated E, indicating that detection of surface Fc receptors reached a level where no more cells participated in the reaction in spite of increasing the sensitization of EA.

Of interest is the observation that the rat tumor and the bladder carcinoma gave the same strength of reaction with tissue sections and with cells in suspension, while the other two tumors showed lower reactivity using cell suspensions

Table 2 shows the results obtained in tests with smears of the cell fractions of the rat tumor. Sediments 1 and 2 had much the same activity as the sections, while sediment 3 (microsomal or membrane rich fraction)gave repeatedly 3 to 4 steps lower activity These data suggested that the receptors might also be present on internal membranes. This assumption was corroborated by the results of the following experiments Tumor cells were treated with neuraminidase to reveal all receptors on the surface of the cells (10). The percent EA rosettes (at ½ unit sensitization) increased from 52 to 85. Then the surface receptors were eluted by suspending the cells in 0.1 M glycine /HCl buffer pH 2 5 (10^6 cells/ml) at 20° C for 15 min The cells were immediately washed in PBS and tested for Fc receptor activity No rosettes were formed even with EA sensitized with 8 agglutinating units of A (formalinized E) The treated cells were then pelletted by centrifugation and the pellet was frozen. Cryostat sections of the pellet adsorbed

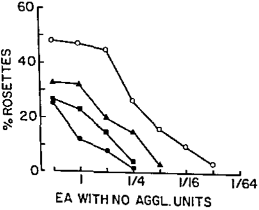


Fig 1 Fc receptors on cells from the rat tumor and three human tumors Percentage of cells in suspension making rosettes with EA sensitized by various amounts of IgG antibodies.

○———○ Walker tumor 256
▲———▲ Bladder carcinoma
●———● Breast carcinoma
■———■ Melanoma

using the trocar technique. Tumors were removed aseptically at various times after transplantation, and necrotic and hemorrhagic portions were removed carefully. Muscle from the hindleg of normal rats and normal rat and human kidney served as control tissues. Tissue not used immediately was placed in phosphate buffered saline, pH 7.2 (PBS), and wrapped in plastic bags to be stored at −70° C.

Single Cell Suspensions

Fresh tissue were minced with iris scissors and suspended in PBS The suspension was centrifuged at $100 \times g$ for 1 min to sediment the coarser particles. The cells in the supernatant were sedimentet by centrifugation at $400 \times g$ for 5 min. They were washed three times in PBS and resuspended in PBS to approximately 2×10^6 cells/ml. The cell suspensions were kept at 4° C.

Subcellular Fractions

Cell fractions were prepared using differential centrifugation at 4° C of tissue homogenate (Servall Omnimixer for 30 s at 4° C) made from fresh or frozen tissue in four parts of 0.25 M sucrose in PBS (7). The homogenate was centrifuged for 30 min at $1{,}500 \times g$ (sediment 1), and the supernatant was centrifuged for 30 min at $12{,}000 \times g$ (sediment 2). The supernatant was again centrifuged at $102{,}000 \times g$ for 60 min (sediment 3) The final supernatant (soluble fraction) was also saved. The sediments (nuclei rich, mitochondria rich, membrane rich) were washed three times in cold PBS. Suspensions of the sediments were made in PBS from the packed sediments. Light microscopic examinations showed that the sediments prepared at different times were fairly uniform

Smears (approximately 5×5 mm) of packed sediments were prepared in the center of large coverglasses After the smears had dried at room temperature, they attached firmly to the glass and could be used in tests described below

Erythrocytes

Sheep erythrocytes were obtained from whole blood kept in equal parts of Alsever's solution Formalinized erythrocytes were prepared following the method described by *Daniel et al* (1)

Demonstration of Fc Receptors in Tissue Sections

The procedure was the same as described earlier using the closed chamber technique (10, 11) Cryostat sections of frozen tissue on large coverglasses were incubated in wet chambers with the indicator cells applied using inverted microculture slides with a single concavity allowing the indicator cells (suspended in PBS containing 2 per cent NACl) to settle on the coverglass for 30 min The slides were then turned and left for detachment of indicator cells from the glass and non-reactive tissue The degree of hemadsorption (3 +, 2 +, 1 +) was recorded microscopically when the glass around the tissue was free of erythrocytes.

The indicator cells (EA) were prepared by sensitizing sheep erythrocytes (E) with rabbit IgG antibodies to E (A) in various amounts expressed as agglutinating units One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of a 1 per cent suspension of E (100 per cent equals E packed for 10 min at $1{,}000 \times g$). The washed EA were used as a 1 per cent suspension (approximately 2×10^8 erythrocytes/ml). The tissue activity was graded according to the reciprocals of the agglutinating units of A on the weakest sensitized EA which gave reaction (8)

For some control experiments E were sensitized with rabbit IgM antibodies.

For staining of the tissue sections after reading sections were fixed in 4 per cent formaldehyde as described earlier (10) and stained with hematoxylin and eosin

Demonstration of Fc Receptors with Cells in Suspension

One or two drops of the cell suspensions were mixed in glass tubes with equal volumes of the proper EA suspensions. The mixtures were left at 20° C for 2 h The cells were then gently resuspended and the percentage of rosette forming cells determined in a counting chamber using light microscopy at 430 ×.

Inhibition Tests

Various lots of 2 per cent EA were mixed with equal volumes of soluble fraction or suspensions of sediments 2 or 3 (Sediment 1 was not used because it made too large clumps with EA) The mixtures were left at 20° C for 30 min One half of the treated EA was washed three times in PBS, the other half was not washed The three EA preparations were then used in tests for Fc receptors with tissue sections and with cells in suspension

Pepsin-digestion

The IgG fraction of rabbit antiserum to E was digested as described by *Stewart et al* (6) The digested product ($F(ab')_2$ fragments) agglutinated E as well as undigested control, but was free of Fc since E sensitized by the digested product were not agglutinated by a high-titered rheumatoid factor serum.

Pretreatment of Tissue Sections

In order to study the effect of various preparations, the sections were handled as described previously (10, 11). In brief, they were covered by the solutions and kept in a moist chamber at 20° C or 37° C for 1 h The treated sections were washed with PBS before they were tested for hemadsorption of EA

EXPERIMENTS AND RESULTS

Table 1 shows the results obtained with the selected tumors tested with our standard method employed in testing for Fc receptors in tissue sections The Walker tumor 256 was one of the strongest reacting tumors (activity 16 to 32); the EA attached to the sections in a diffuse pattern, apparently covering both external and internal structures of the large tumor cells. That the activity of this tumor was dependent on the Fc portion of the IgG molecule bound to the erythrocytes was evident

preparation of membranes involved dilution and prolonged washing, loosely associated components would be lost during the process. Since all three sediments (nuclear, mitochondrial and microsomal/membrane rich fractions) showed receptor activity, it is possible that the receptors are also bound to internal membranes in addition to external membranes. Although each fraction was contaminated with all the other components, it is unlikely that fragments of external membranes should be responsible for all Fc receptor activity in each of the three sediments. Particularly sediment 2, the mitochondria rich fraction, had repeatedly the strongest Fc receptor activity.

That internal membranes possess Fc receptors are also suggested by the results obtained with sections of cells with no surface receptors. Fc receptors were easily demonstrated in the sectioned cells. Results with adsorption of EA to sections of the tumors support this conclusion as EA attached to the tissue in a diffuse pattern. Even with such a large indicator as an erythrocyte, this was easy to distinguish in the rat tumor which contained many large tumor cells. Apparently the same cells may carry both external and internal receptors, suggesting that the receptors are produced by, and can be released from the cells. Recently it has been shown that Fc receptor-like material is indeed shed from some human lymphoblastoid cell lines (5).

The results obtained with tumor cells in suspension is in accord with earlier results that the rosette formation is dependent on the degree of sensitization of EA. In addition, neuraminidase treatment reveals receptors on more cells. This indicates that the Fc receptor is not a property of a distinct subpopulation of cells (10).

Our data do not allow definite conclusions concerning the quantitative distribution on the various membranes. Further detailed studies are needed, and electron microscopic investigations with enzymes and other markers are currently in progress. However, since the two concentrated subcellular fractions (mitochondrial, and microsomal) did not show a significantly increased activity, the Fc receptor is tentatively present in highest numbers on the cell surface. Apparently there are no differences in avidity of the receptors on the various membranes as the fractions gave parallel results with EA sensitized to various degrees. Further, the results of inhibition experiments using EA incubated in membrane suspension did not suggest any differences in avidity.

These findings have important implications for the selection of test systems for detection of Fc receptors, particularly since other data (9) indicate that IgG can be bound to the surface Fc receptor *in vivo* and thereby block the *in vitro* reactivity. Whether the cells carrying Fc receptors can phagocytize IgG which then might be bound to internal membranes and block these receptors also remains to be investigated. This possibility is supported by some of the results reported by *Witz* (12), who found that IgG could be eluted from the mitochondria rich fractions of murine tumors, but in amounts 2 to 3 times less than the amount eluted from corresponding membrane rich fractions. Therefore, both external and internal receptors might be covered *in vivo*, but one would suspect that the surface receptors can most easily be blocked or covered and therefore be quantitatively diminished or even undetectable on some cells *in vitro*.

The localization of the Fc receptor in tumors to external and probably also to internal membranes is similar to the localization of the receptors for cytophilic antibodies on and in monocytes and macrophages (2). The only apparent difference being that the latter receptors have higher avidity for uncomplexed IgG. In this respect the tumor Fc receptor is more similar to the Fc receptor on B lymphocytes than on monocytes; however, at present it is not possible to definitely distinguish between these receptors on the three cell types (4, 10). On the basis of the results obtained with a variety of mouse connective and epithelial tissue tumors, *Kerbel et al.* (3) concluded that the cells bearing Fc receptors were host cells, many of them perhaps being macrophages in an activated state. Experiments are currently in progress to further characterize such cells in human tumors.

This work was supported by a grant from the *Women's Auxiliary to the Veterans of Foreign Wars* and by the *Mid-America Immunotherapy and Surgical research Foundation*. The authors wish to thank Mrs. *Carol Boettcher* and Mrs. *Eli Jensen* for excellent technical assistance.

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E sensitized by ½ or more units of A, indicating that internal receptors were still made available after sectioning.

Experiments were then performed to study the ability of the mitochondria rich and membrane rich preparations from the murine tumor (sediments 2 and 3) to inhibit the reaction of EA with tissue sections of the same tumor. EA sensitized with 1, $^1/_2$, $^1/_4$, or $^1/_8$ agglutinating units were incubated with equal volumes of suspensions of the sediments at concentrations from 1 to 8 per cent as described above. The results obtained with sediment 2 treated, unwashed EA are shown in Table 3. The highest concentrations of sediment inhibited the reactions with all EA preparations and the lowest concentration still inhibited the weakest sensitized EA. Similar results were obtained with sediment 3. Washed, treated EA gave almost the same inhibition patterns, indicating that the inhibition was the result of a blocking of the reactive sites on the EA. This was further substantiated by the finding that sections which had not adsorbed the treated EA gave strong reactions when tested with untreated EA.

Whether the inhibitory material in the membrane fractions had specificity for EA was investigated in absorption experiments. Aliquots of a 2% suspension of sediment 2 were incubated with equal volumes of packed formalinized E sensitized with 4 agglutinating units of IgG, IgGF(ab')$_2$ and IgM, respectively. After centrifugation, the supernatants were used in inhibition experiments. IgGEA exhausted the inhibiting effect, while the other EA preparations did not influence the activity In control experiments using cell fractions of rat muscle and kidney no inhibition was obtained

The soluble fraction also inhibited, although weakly, and the effect disappeared at a 1 in 4 dilution (Table 3). Since similar effect was obtained with the soluble fractions of the normal rat organs, further experiments with the soluble fraction were not performed in this series.

The cell fractions from the three human tumors showed similar patterns of reactivity as the rat tumor in tests with smears. Because of limited amounts of material available, inhibition experiments were performed with selected EA preparations only. However, the patterns of reactions obtained were similar to those obtained with the rat tumor.

Many human tumors could not be tested for cell surface receptors because mechanical disruption of tissue did not give single cells. However, smears of the tumor sediments again gave results comparable to those described for the other tumors.

Single cells of the rat tumor were also studied in inhibition test with EA incubated in suspension of the various cell sediments or in the soluble cell fraction. The percentage of rosetting cells with EA at ½ unit sensitization was reduced from 47 to less than 10 per cent with sediment concentrations of 4 and 8 per cent In suspensions showing inhibition of rosette formation with tumor cells, the EA made separate »rosettes« around smaller or larger sediment particles. In control experiments using cell fractions of normal rat muscle and kidney no reduction in number of rosettes was recorded even with 8 per cent suspension

TABLE 2. *Adsorption of EA Sensitized by Various Amounts of IgG Antibodies to Smears of Cell Sediments of Walker Tumor 256*

Preparation	EA with no. of agglutinating units of A						
	2	1	$^1/_2$	$^1/_4$	$^1/_8$	$^1/_{16}$	$^1/_{32}$
Tissue sections	3+	3+	3+	2+	2+	1+	–
Sediment							
1	3+	3+	2+	1+	–	–	–
2	3+	3+	3+	2+	1+	–	–
3	3+	1+	±	–	–	–	–

TABLE 3. *Adsorption of EA to Sections of Walker Tumor 256. Effect of Preincubation of EA with Various Amounts of Cell Fractions of the Same Tumor*

Pretreatment with	EA with no of agglutinating units of A			
	1	$^1/_2$	$^1/_4$	$^1/_8$
Sediment 2, % suspension				
8	–	–	–	–
4	2+	–	–	–
2	2+	1+	–	–
1	3+	3+	2+	1+
Soluble fraction				
Undil.	2+	1+	–	–
1 in 4	3+	2+	2+	1+
PBS	3+	3+	2+	2+

DISCUSSION

The results obtained with tumor cells in suspension clearly show that the receptors for IgGFc in tumors are available at the cell surface. That this activity could be traced in the various sediments of subcellular fractions indicate that the receptors are bound firmly to the cell membranes. Because

eparation of membranes involved dilution and olonged washing, loosely associated components ould be lost during the process. Since all three diments (nuclear, mitochondrial and microsomal/ embrane rich fractions) showed receptor activity, is possible that the receptors are also bound to ternal membranes in addition to external memb- ines Although each fraction was contaminated ith all the other components, it is unlikely that agments of external membranes should be respon- ible for all Fc receptor activity in each of the three diments Particularly sediment 2, the mitochon- ria rich fraction, had repeatedly the strongest Fc eceptor activity

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Our data do not allow definite conclusions concerning the quantitative distribution on the various membranes Further detailed studies are needed, and electron microscopic investigations with enzymes and other markers are currently in progress However, since the two concentrated subcellular fractions (mitochondrial, and microso- mal) did not show a significantly increased activity, the Fc receptor is tentatively present in highest numbers on the cell surface. Apparently there are no differences in avidity of the receptors on the various membranes as the fractions gave parallel results with EA sensitized to various degrees. Further, the results of inhibition experiments using EA incubated in membrane suspension did not suggest any differences in avidity

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IMMUNO-SUPPRESSIVE EFFECT OF HUMAN MACROPHAGES

I. Evidence for an Unstable Soluble Factor(s) Inhibiting DNA Synthesis and Proliferation in PHA-Stimulated Lymphocytes

G. UNSGAARD

University of Trondheim, Department of Medicine, Section for Haematology and Immunology, Trondheim, Norway

Unsgaard, G. Immuno-suppressive effect of human macrophages. I Evidence for an unstable factor(s) inhibiting DNA synthesis and proliferation in PHA-stimulated lymphocytes. Acta path microbiol. scand Sect C, 86 179–186, 1978

Macrophages obtained by culturing blood monocytes *in vitro* for 8 days showed capacity to inhibit DNA synthesis and cell proliferation in PHA-stimulated lymphocytes separated from the macrophages by a membrane with pore size 0 2 μm The DNA synthesis was measured as ^{3}H thymidine incorporation and the proliferation as cell counts The depression was reduced when the distance between macrophages and lymphocytes was increased concomitant with increased culture volume Heat-killing of macrophages abolished their lymphocyte depressing capacity. Full inhibitory effect was established within 4 hours when lymphocytes were cultured in the proximity of macrophages The effect was blocked by molecular filtration membranes with nominal molecular weight limits (nmwl) 1,000 and 10,000 whereas membranes with nmwl 25,000 only partially blocked the effect No inhibitory effect was registered in the supernatants from macrophage-depressed lymphocyte cultures. Presumably the lymphocyte depressing effect is mediated via an unstable soluble factor(s)

Key words Macrophages, lymphocytes, ^{3}H thymidine, PHA, immuno-suppression.

G Unsgaard, Trondheim Regional Hospital, Department of Medicine, Section for Haematology and Immunology, N-7000 Trondheim, Norway

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There is convincing evidence indicating that mononuclear phagocytes play an important role in immunoregulation. Their role in induction of antibody response (12, 24, 30), cell-mediated immunity (9, 10, 11, 17, 26, 27) and augmentation of production of lymphocyte mediators (19) is well established. Moreover, there are numerous reports which reveal an immunodepressive role of mononuclear phagocytes (1, 6, 13, 15, 16). However, data regarding the mechanisms of this depressive effect have been conflicting. Some investigators claim that cell contact is essential, since culture supernatants fail to inhibit lymphocyte proliferation (14, 28, 29). Others have found that macrophage culture supernatants can inhibit lymphocyte proliferation (21, 34). However, supernatants are usually less potent inhibitors than live macrophages.

Studies on human systems of the immunoregulatory role of mononuclear phagocytes are scarce. In a human *in vitro* system, we have shown that mononuclear phagocytes play both immuno-suppressive and immuno-inductive roles (31). The depressive effect of mononuclear phagocytes was related to the differentiation of monocytes to macrophages (32) and to the ratio of mononuclear phagocytes to lymphocytes. A high ratio inhibited DNA synthesis more than a low ratio, and the macrophage was a more potent inhibitor than the monocyte. Evidence is presented in this paper which

indicates that the mechanism for depression of DNA synthesis in stimulated lymphocytes is mediated via an unstable soluble factor(s) secreted by the mononuclear phagocytes.

MATERIALS AND METHODS

Separation techniques. Venous blood obtained by venipuncture of healthy, adult human volunteers was defibrinated. Mononuclear cells were separated by centrifugation on Lymphorep (Nyegaard & Co A/S, Oslo, Norway), as described previously (31, 32)

Culture medium The experiments were performed in culture medium consisting of 80% RPMI 1640 (Gibco Bio-Cult, Glasgow, Scotland) and 20% pooled human AB serum, supplemented with 0.1 mg l-glutamin per ml Gentamycin was added to RPMI 1640 to a concentration of 40 mg per l. Since AB serum was difficult to obtain, the macrophages were precultured in a medium consisting of 25% pooled human A^+ serum and 75% RPMI 1640.

Macrophage cultures The mononuclear cells were suspended in culture medium and adjusted to 4×10^6 cells per ml. Two and a half ml of this suspension was incubated in plastic petri dishes (Nunclon, Ø 50 mm) for 90 min. The culture medium with non-adherent cells was removed and 2.5 ml fresh medium added The culture medium was changed after 1 and 4 days. Experiments were started when the macrophages had been cultured for 8 days.

Lymphocyte cultures. The culture medium with non-adherent cells removed after the 90 min attachment period was considered to be a fairly pure source of lymphocytes This cell suspension contained about 4% cells which will attach on renewed incubation in petri dishes (31). The lymphocyte suspension was adjusted to 10^6 cells per ml culture medium Two and a half ml was added per dish In some experiments lymphocytes were precultured in Kimax tubes for 3 days and then transferred to petri dishes

Technique for separating lymphocytes from macrophages by a membrane system Polycarbonate membranes (Uni-Por, Bio-Rad Laboratories, Richmond, USA), pore size 0.2 µm and diameter 50 mm, were glued to glass rings with silicon rubber (Silite 100, Devcon corp, Mass, USA), thus forming membrane chambers (compartment A, Fig 1) The inner and outer diameters of these rings were 43 mm and 47 mm respectively, and the height 15 mm Glass rings with the same outer and inner diameter and heights ½ mm, 2 mm and 4mm, were placed in the bottom of the culture dishes The membrane chambers and the rings were sterilized by autoclaving. Before the glass rings were placed in the culture dishes, the macrophages were romoved by a rubber policeman from a 7 mm wide zone along the edges of the dishes, to make sure that the glass rings did not destroy macrophages during the experiments. The membrane chambers were mounted on these rings, thus forming a double chamber (Fig 1) Compartment B was filled with different volumes of medium according to the heights of the lower rings. The suitable volumes were 2 ml, 4.5 ml and 8 ml when the heights of the rings were ½ mm, 2 mm and 4 mm respectively. The membrane chambers were filled with 2.5 ml of lymphocyte suspension.

In some experiments, Pellicon membranes (Millipor Corp., Mass., USA) produced for molecular filtration were used to make membrane chambers The membranes had different nominal molecular weight limits (nmwl). PSAC (nmwl 1,000), PTGC (nmwl 10,000), PSED (nmwl 25,000). The diameter was 47 mm.

PHA stimulation Lyophilized phytohaemagglutinin from Gibco reconstituted with 10 ml distilled water was added in a quantity of 50 µl per culture dish or Kimax tube. This dose gave optimal DNA synthesis in the lymphocytes (32)

Assay of DNA synthesis ^{3}H thymidine (sp act. 26 Ci/nmol) was added in a concentration of 0 5 µCi per ml culture medium The isotope was present in the culture medium during the last 4 hours before harvesting of cells At the end of the culture period, lymphocytes were carried over from membrane chambers or from the bottom of culture dishes to tubes by transferring medium with cells and washing vigorously with 10 ml cold isotonic saline which was also transferred to the tubes

Cells were harvested by a Titertek multiple cell harvester (Flow, Scotland). The filters were applied to vials and dried overnight at 40° C, and the radioactivity incorporated was counted in a liquid scintillation counter (Packard, Tri-Carb) for 3 min. The data presented are based on counts per min (c p.m).

Proliferation estimated by cell counts Lymphocytes were transferred to tubes as already described However for washing, Hanks' BSS was used instead of isotonic saline. The tubes were centrifuged at 800 G for 10 min, and the cells resuspended in 1 ml RPMI 1640. The cells were counted in an electronic particle counter (Coulter Counter, Model Fn) and in a haemocytometer.

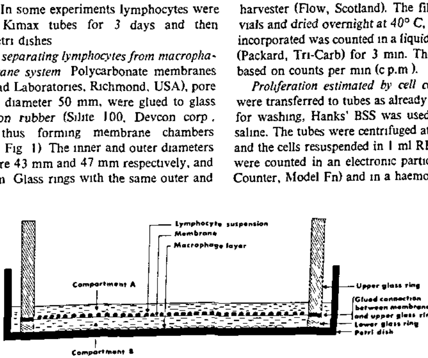


Fig. 1 A drawing of the double chamber system The figure is described in the text.

TABLE 1 *Influence of Macrophages on DNA Synthesis in PHA- Stimulated Lymphocytes Effect of Macrophage/ Lymphocyte separation*

Degree of macrophage/lymphocyte separation	^{3}H thymidine incorporation With M [a)] c.p.m. × 10^{-3}	Without M (control) c.p.m. × 10^{-3}	With M Percentage of control
Lymphocytes sedimented on macrophages	20 ± 4	490 ± 21	4
Lymphocytes sedimented on a membrane[b)] separated from the macrophage layer by a ½ mm glass ring	97 ± 14	485 ± 32	20
Lymphocytes sedimented on a membrane separated from the macrophage layer by a 2 mm glass ring	235 ± 49	509 ± 52	46
Lymphocytes sedimented on a membrane separated from the macrophage layer by a 4 mm glass ring	400 ± 59	554 ± 46	72
Lymphocytes sedimented on a membrane, PHA not added to the culture		2	

The lymphocytes were cultured in contact with or in the proximity of macrophages for 96 hours
The values are means ± SEM of six experiments
[a)] M – macrophages
[b)] Polycarbonate membrane (Uni-Por) with pore size 0.2 μm

RESULTS

Effect of separating lymphocytes from macrophages by a membrane The lymphocytes were either sedimented on the macrophages or separated from the macrophages by a membrane, as shown in Fig. 1 PHA was added and the cultures were incubated for 96 hours DNA synthesis and proliferation of the lymphocytes were investigated (Tables 1 and 2).

DNA synthesis and cell proliferation were strongly inhibited when lymphocytes were sedimented on macrophages. Under these conditions, the lymphocytes showed a tendency to cluster around the macrophages

Separating lymphocytes from macrophages by a polycarbonate membrane did not abolish the inhibition of the DNA synthesis When the

TABLE 2 *Influence of Macrophages on Proliferation in PHA-Stimulated Lymphocytes Effect of Macrophage/ Lymphocyte Separation*

Degree of macrophage/lymphocyte separation	Cell count in Coulter Counter × 10^{-6}		Cell count in haemocytometer × 10^{-6}	
	With M[a)]	Without M (control)	With M	Without M (control)
Lymphocytes sedimented on macrophages	1.2 ± 0.2	5.0 ± 0.8	0.7 ± 0.1	4.9 ± 0.5
Lymphocytes sedimented on a membrane[b)] separated from the macrophage layer by a ½ mm glass ring	1.6 ± 0.1	5.6 ± 0.5	1.8 ± 0.1	5.2 ± 0.1
Lymphocytes sedimented on a membrane separated from the macrophage layer by a 2 mm glass ring	2.4 ± 0.3	6.2 ± 0.8	2.1 ± 0.3	6.1 ± 1.0
Lymphocytes sedimented on a membrane separated from the macrophage layer by a 4 mm glass ring	3.1 ± 0.4	6.1 ± 0.8	3.1 ± 0.6	5.3 ± 0.6
Lymphocytes sedimented on a membrane, PHA was not added to the culture		2.2 ± 0.5		2.0 ± 0.5

The lymphocytes were cultured in contact with or in the proximity of macrophages for 96 hours
The values are means ± SEM of six experiments.
[a)] M – macrophages
[b)] Polycarbonate membrane (Uni-Por) with pore size 0.2 μm

TABLE 3. *Effect of Heat-Killed Macrophages on DNA Synthesis and Proliferation i PHA- Stimulated Lymphocytes Sedimented on a Membrane[a)] Separated from Macrophages by ½ mm Glass Ring*

Macrophages	^3H thymidine incorp. c.p.m. $\times 10^{-3}$	Cell counts $\times 10^{-6}$ Coulter Counter	Haemocytometer
Untreated	74 ± 16	1.9 ± 0.5	2.2 ± 0 4
Heat-Killed (56° C for 30 min)	415 ± 34	4.5 ± 0.9	4 6 ± 0 8
Not present (control)	522 ± 37	5.1 ± 0.8	5.2 ± 0.8

The lymphocytes were cultured in the proximity of macrophages for 72 hours. The values are means ± SEM of six experiments.
a) Polycarbonate membrane, pore size 0.2 μm.

membrane chambers were mounted on ½ mm high rings, the DNA synthesis was still inhibited to 20 per cent of the controls. The inhibition was reduced to 46 per cent and 72 per cent of the controls when the height of the rings was increased to 2 mm and 4 mm, respectively (Table 1).

In some experiments four times the usual concentration of ^3H thymidine was added, but this did not influence the degree of inhibition.

The effect of macrophages on lymphocyte proliferation was similar to the effect on DNA synthesis (Table 2).

Effect of heat-killing macrophages. Macrophages were pretreated at 56° C for 30 min. This totally abolished the capacity of macrophages to phagocytize candida, as evaluated by phase contrast microscopy and registration of uptake of ^{125}I-labelled *Candida albicans* (33). No morphological damage to the cell surface was observed. The trypan blue dye test showed that heat-treated cells took up this dye (>90%), thus indicating that the cells were no longer viable.

The effect of heat-killing on macrophage-mediated inhibition of DNA-synthesis and cell proliferation was tested in the double chamber system. Lymphocyte suspension with PHA was applied to membrane chambers (polycarbonate membranes) mounted on ½ mm high glass rings placed in petri dishes with normal macrophages, heat-killed macrophages or without macrophages. The lymphocytes were harvested 96 hours later. The heat-killing reduced markedly the capacity of macrophages to inhibit DNA synthesis and cell proliferation (Table 3). The inhibition of DNA synthesis was reduced from 14 per cent of the controls by normal macrophages to 80 per cent of the controls by heat-killed macrophages. The inhibition of cell proliferation was reduced similarly.

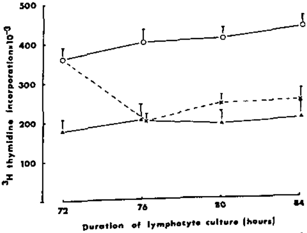


Fig. 2 ^3H thymidine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in cultured dishes with or without M. (▲—▲) with M; (O—O), without M, (×—×), without M for 72 hours before transfer to dishes with M. The values are means ± SEM of five experiments.

Kinetics of macrophage-mediated inhibition of DNA synthesis in PHA-stimulated lymphocytes tested in the double chamber system. Lymphocyte suspensions with PHA were applied to membrane chambers (polycarbonate membranes) mounted on ½ mm high rings placed in culture dishes with or without macrophages. Seventy-two hours later some of the membrane chambers were transferred from dishes without macrophages to dishes with macrophages. This caused an immediate depression of lymphocyte DNA synthesis to the level of controls exposed to macrophages from the start of the experiment (fig. 2).

Lack of inhibitory effect by culture supernatants from depressed cultures Supernatants were prepared from media obtained from compartment B (Fig.

TABLE 4 *^3H thymidine Incorporation in PHA-Stimulated Lymphocytes Suspended in Culture Supernatants from Macrophage Depressed Lymphocyte Cultures*

Supernatants tested	Incubation time. test lymphocytes + supernatants	^3H thymidine incorporation c p m. $\times 10^{-3}$
Supernatants from 48-h cultures of lymphocytes with macrophages in compartment B	24 h	493 ± 75
Supernatants from 48-h cultures of lymphocytes without macrophages	24 h	522 ± 45
Test lymphocytes resuspended in fresh medium		475 ± 73
Supernatants from 4-h cultures of lymphocytes with macrophages in compartment B	4 h	348 ± 26
Supernatants from 4-h cultures of lymphocytes without macrophages	4 h	342 ± 24

The supernatants were taken from compartment B in the double chamber system. The membrane chambers (polycarbonate membranes) were mounted on ½ mm high rings The values are means ± SEM of four experiments carried out in triplicate

1) in the double chamber system. The membrane chambers (polycarbonate membranes) were mounted on ½ mm high rings. At the time of supernatant preparation, the lymphocytes in the membrane chambers had been cultured with PHA for 48 hours either in the presence of macrophages from start of culture or during the last 4 hours only. In both cases DNA synthesis in the membrane chamber lymphocytes was depressed (fig. 2).

The supernatants of these depressed cultures were immediately tested for effect on DNA synthesis in other stimulated lymphocytes (test lymphocytes). These test lymphocytes were precultured for 72 hours before they were resuspended in culture supernatants and cultured in dishes for 4 or 24 hours

The supernatants showed no inhibitory effect (Table 4).

Block of macrophage-mediated inhibition when lymphocytes were separated from macrophages by Pellicon membranes (Millipor). Pellicon membranes with different nominal molecular weight limits (nmwl) were used for the membrane chambers, which were mounted on 1 mm high glass rings (Fig. 1). Medium volume in compartment B was 3.5 ml. PHA-stimulated lymphocytes precultured for 72 hours were transferred to the membrane chambers placed in culture dishes with or without macrophages. The cells were harvested 8 hours later. Polycarbonate membranes were used for inhibition control.

The macrophage-mediated inhibitory effect on DNA synthesis in stimulated lymphocytes was blocked (Table 5) by PSAC (nmwl 1,000) and PTGC (nmwl 10,000), and partially by PSED (nmwl 25,000).

TABLE 5 *Influence of Macrophages on DNA synthesis in PHA-Stimulated Lymphocytes Effect of Separating Lymphocytes from Macrophages by Membranes with Different Pore Size*

Membrane	^3H thymidine incorporation		
	With M c p m $\times 10^{-3}$	Without M (controls) c p m $\times 10^{-3}$	With M Percentage of control
PSAC[a] (nmwl 1,000)	157 ± 63	163 ± 57	96
PTGC[a] (nmwl 10,000)	283 ± 42	287 ± 52	99
PSED[a] (nmwl 25,000)	210 ± 49	268 ± 56	78
Polycarbonate membrane, pore size 0 2 µm	182 ± 40	340 ± 40	54

The membrane chambers were mounted on 1 mm high rings in culture dishes with or without macrophages Culture time 8 hours The values are means ± SEM of six experiments.

[a] Molecular filtration membranes (Pellicon, Millipor); with statement of nominal molecular weight limits.

TABLE 3. *Effect of Heat-Killed Macrophages on DNA Synthesis and Proliferation i PHA-Stimulated Lymphocytes Sedimented on a Membrane[a] Separated from Macrophages by ½ mm Glass Ring*

Macrophages	^{3}H thymidine incorp. c.p.m. × 10^{-3}	Cell counts × 10^{-6}	
		Coulter Counter	Haemocytometer
Untreated	74 ± 16	1.9 ± 0.5	2.2 ± 0.4
Heat-Killed (56° C for 30 min)	415 ± 34	4.5 ± 0.9	4.6 ± 0.8
Not present (control)	522 ± 37	5.1 ± 0.8	5.2 ± 0.8

The lymphocytes were cultured in the proximity of macrophages for 72 hours. The values are means ± SEM of six experiments.

[a] Polycarbonate membrane, pore size 0.2 µm.

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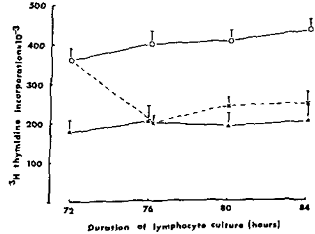


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The effect of heat-killing on macrophage-mediated inhibition of DNA-synthesis and cell proliferation was tested in the double chamber system. Lymphocyte suspension with PHA was applied to membrane chambers (polycarbonate membranes) mounted on ½ mm high glass rings placed in petri dishes with normal macrophages, heat-killed macrophages or without macrophages. The lymphocytes were harvested 96 hours later. The heat-killing reduced markedly the capacity of macrophages to inhibit DNA synthesis and cell proliferation (Table 3). The inhibition of DNA synthesis was reduced from 14 per cent of the controls by normal macrophages to 80 per cent of the controls by heat-killed macrophages. The inhibition of cell proliferation was reduced similarly.

Kinetics of macrophage-mediated inhibition of DNA synthesis in PHA-stimulated lymphocytes tested in the double chamber system. Lymphocyte suspensions with PHA were applied to membrane chambers (polycarbonate membranes) mounted on ½ mm high rings placed in culture dishes with or without macrophages. Seventy-two hours later some of the membrane chambers were transferred from dishes without macrophages to dishes with macrophages. This caused an immediate depression of lymphocyte DNA synthesis to the level of controls exposed to macrophages from the start of the experiment (fig 2).

Lack of inhibitory effect by culture supernatants from depressed cultures. Supernatants were prepared from media obtained from compartment B (Fig.

bably mediated via an unstable soluble factor(s) (20). He based this assumption on the contradictory results of authors searching for soluble inhibitory factors. In favour of this hypothesis is also the physically close association between macrophages and lymphocytes, seen both *in vivo* and when co-culturing the cells *in vitro* (26), thereby enhancing the effectiveness of unstable molecules just leaving the macrophages. Our results are in agreement with *Nelson*'s assumption, giving evidence that the predominant mechanism for lymphocyte depression by macrophages in a human system is mediated via an unstable soluble factor(s)

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In order to get an indication of molecule transport through the different membranes, transport of some tracer molecules was tested. Three and a half ml of isotonic saline was added to compartment A (Fig. 1) and 3 ½ ml of a solution of tracer in isotonic saline to compartment B. The tracers were creatinine (100 μg/ml), ^{57}Co-labelled vitamin B_{12} (100 μg/ml) with final specific activity 0.50 μCi/l and ^{14}C-labelled inulin (4 mg/ml) with final specific activity 10 μCi/l. Eights hours later samples were taken from the two compartments and the distribution of tracers was registered.

The results showed that creatinine (mol.wt. 113) passed easily through all the membranes. The transport of vitamin B_{12} (mol.wt. 1335) and inulin (mol.wt. 5200) was blocked by PSAC and PTGC, but equilibrium was established over PSED and the polycarbonate membrane (pore size 0.2 μm).

DISCUSSION

The results of several experiments in animal systems have shown that macrophages or macrophage products depress lymphocyte activity (1, 2, 6, 14, 21, 34). Human monocyte-derived macrophages also have a depressive effect on DNA synthesis in stimulated lymphocytes (32). The inhibition is non specific. Autologous and allogeneic lymphocytes are equally depressed, and the inhibition is similar for lymphocytes stimulated by PPD (purified tuberculin), allogeneic lymphocytes or PHA.

A principal question is how this inhibition is mediated. Is cell contact necessary, or is it mediated by soluble factor(s)? The effect of separating macrophages and lymphocytes by a polycarbonate membrane indicates that a factor or factors produced by the macrophages are important for this type of inhibition. In these experiments, about half of the macrophages were removed from the culture dishes to clear an area for the glass rings Nevertheless, lymphocyte DNA synthesis in these cultures was depressed to 20 per cent of the controls. A cytotoxic effect of membrane or glue can be excluded, because lymphocyte controls in membrane chambers responded just as well to PHA as those in petri dishes. Furthermore, experiments performed in membrane chambers kept together with pressure instead of glue gave the same results

In murine systems, macrophage factors depressing lymphocyte response have been reported by many authors (2, 20). In some systems, the factor(s) depressing ^{3}H thymidine and ^{125}I-deoxy-uridine incorporation was shown to be non-labelled nucleotides competing with labelled nucleotide The non-labelled nucleotides were probably degradation products of dying cells (5, 6, 22, 23). In those systems proliferation was not affected. However, it must be borne in mind that excess of thymidine will also inhibit cell proliferation (3, 7).

In the system of human macrophages and lymphocytes, supernatants from depressed cultures did not have inhibitory effect on DNA synthesis in other stimulated lymphocytes. The macrophages may thus secrete a factor that is inactivated in less than 4 hours, or there may be an elimination of helping factors from the lymphocyte suspension. It excludes possible explanations like malnutrition and blockage by non-labelled thymidine.

The concept of helping factor elimination as a mechanism for macrophage-mediated depression of lymphocyte response is based on the results of *Ptak & Gershon*, which showed that heat-killed macrophages depressed DNA synthesis in stimulated lymphocytes (8, 25). The macrophages were heat-treated at 56° C for 30 min. They showed that this did not destroy receptors for cytophilic antibodies When human macrophages were treated in the same way, they lost most of their inhibitory effect Therefore, assuming that human macrophage receptors are as heat-stable as murine macrophage receptors, elimination of helping factors is only of minor importance in a system of human cells if it contributes at all

The most likely interpretation of the experiments is that macrophages secrete continuously an unstable factor(s). The concept of unstable factors secreted by macrophages is not new, having been discussed in relation to murine systems (4, 18). The extremely unstable factor described by *Melsom et al* (18) had a lytic effect on mouse erythrocytes, whereas the experiments of *Currie & Basham* (4) revealed a more stable factor lysing sarcoma cells The effect of increasing the distance between the macrophage and the lymphocyte population might lead to the conclusion that the diffusion time is critical for inactivation In that case the lymphocyte depressing factor(s) would also be extremely unstable Such a conclusion cannot be drawn, since increasing distance is accompanied by increasing volume of medium, giving a dilution effect.

The Pellicon membrane experiments confirm that soluble factors are involved. However, little can be said about the molecular weight (mol wt.) of the factor(s) from these experiments. In order to estimate mol.wt. by means of tracer molecules, it is necessary to know how quickly the factor(s) is inactivated and to know if secretion of factor(s) depressing lymphocytes is dependent on a signal from lymphocyte mediators

In a review on the non-specific immunoregulation of macrophages, Nelson suggested that the mechanism for lymphocyte depression was pro-

IMMUNO-SUPPRESSIVE EFFECT OF HUMAN MACROPHAGES

II. Influence of Macrophages on the Kinetics of DNA, RNA and Protein Synthesis in PHA-Stimulated Lymphocytes

G UNSGAARD

University of Trondheim, Department of Medicine, Section for Haematology and Immunology, Trondheim, Norway

Unsgaard, G Immuno-suppressive effect of human macrophages. II Influence of macrophages on the kinetics of DNA, RNA and protein synthesis in PHA-stimulated lymphocytes. Acta path. microbiol. scand Sect C, *86* 187–192, 1978

Human macrophages obtained by culturing blood monocytes *in vitro* for 8 days release a soluble factor(s) inhibiting DNA synthesis and cell proliferation in PHA-stimulated lymphocytes Synthesis of RNA and protein was strongly inhibited in PHA-stimulated lymphocytes sedimented on macrophages, as well as in lymphocytes separeted from the macrophages by a membrane, thus demomatrating that inhibition of RNA and protein synthesis was also mediated by a soluble factor(s) The kinetics of the factor-mediated inhibition showed that RNA synthesis was readily inhibited, whereas there was a delay in the inhibition of protein synthesis, thus indicating that the inhibition of protein synthesis and cell proliferation is a result of a blocking effect on the nucleic acid synthesis The kinetics of DNA and protein synthesis in lymphocytes relieved from macrophage-mediated inhibition indicate a cytostatic effect.

Key words Macrophages, lymphocytes; immuno-suppression, RNA-synthesis. protein synthesis, cytostatic factor(s)

Geirmund Unsgaard, Trondheim Regional Hospital, Department of Medicine, Section for Haematology and Immunology, N-7000 Trondheim, Norway

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Accumulating data, mainly in animal systems, show that macrophages have the ability to depress DNA synthesis in stimulated lymphocytes (1,4,7,-8,9) In a human system, we found that blood-derived monocytes developed capacity to inhibit DNA synthesis in stimulated lymphocytes when differentiating to macrophages *in vitro* (14, 15) In a preceding article, results were presented which indicate that this inhibition is mediated by an unstable soluble factor(s) (13)

The aim of this work was to study how lymphocytes were affected by macrophage-mediated depression One question was how RNA and protein synthesis were affected A second question was whether the factor-mediated depression was due to a cytostatic or a cytolytic effect. The results indicate a cytostatic mechanism mediated via an effect on nucleic acid synthesis

MATERIALS AND METHODS

Cells and culture conditions The separation technique and the preparation of lymphocyte and macrophage cultures have been described in a preceding paper (13) Briefly, mononuclear cells isolated from venous blood by centrifugation on Lymphoprep (Nyegaard & Co A/S, Oslo, Norway) were suspended in medium consisting of 20 per cent pooled human AB serum and 80 per cent RPMI 1640 (Gibco Bio-Cult, Glasgow, Scotland) The suspension was incubated for 90 min in culture dishes (Nunclon Ø 50 mm) at a concentration of 4×10^6 cells per ml before removal of non-adherent cells. The

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TABLE 2 *Influence of Macrophages on ³H Uridine Incorporation in PHA-Stimulated Lymphocytes Sedimented on the Macrophage Layer*

Duration of lymphocyte culture	³H uridine incorporation in c.p.m × 10⁻³			
	Incorporation in lymphocytes			Incorporation in macrophages co-cultured with PHA-stim lymphocytes
	PHA-stimulated		Non-stimulated	
	With M[a]	Without M	Without M	
24 h	18 ± 7	55 ± 15	3 ± 1	127 ± 15
48 h	26 ± 5	211 ± 30	3 ± 1	80 ± 12
72 h	20 ± 8	264 ± 32	2 ± 0	96 ± 15
96 h	31 ± 14	301 ± 38	2 ± 0	83 ± 16

This table shows the results of five ³uridine incorporation experiments ± SEM carried out exactly like the ³H leucine incorporation experiments presented in Table 1

macrophages on ³H leucine incorporation in lymphocytes.

The lectin-induced RNA synthesis in lymphocytes, as measured by ³H *uridine incorporation*, increased more abruptly than the protein synthesis during the first 48 hours of culture (Table 2). The rate of ³H uridine incorporation increased 20-fold compared to unstimulated controls during the first 24 hours A further 4-fold increase was seen during the next 24 hours, but there was only a minor increase during the subsequent 48 hours.

Macrophages had a strong inhibitory effect on ³H uridine incorporation in stimulated lymphocytes. However, the depression was not total, since ³H uridine incorporation in stimulated lymphocytes co-cultured with macrophages was about 10-fold higher than in unstimulated controls.

The incorporation of ³H leucine and ³H uridine in macrophages co-cultured with lymphocytes was considerable, but did not seem to lead to isotope depletion, because the sum of the incorporation in macrophages and co-cultured lymphocytes was far less than the maximum incorporation registered in stimulated lymphocytes cultured alone.

Protein and RNA synthesis in PHA-stimulated lymphocytes separated from the macrophages by a membrane. Lymphocyte suspension with PHA was added to membrane chambers placed in culture dishes with or without macrophages. Seventy-two hours after start of the experiment, some membrane

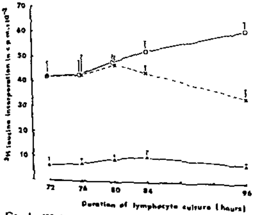


Fig 1 ³H leucine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M (▲—▲), with M, (O—O), without M, (x—x), without M for 72 hours before transfer to dishes with M The values are means ± SEM of five experiments

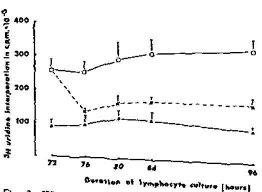


Fig 2. ³H uridine incorporation in PHA-stimulated lymphocytes cultured in *membrane chambers* mounted on ½ mm high rings placed in culture dishes with or without M. (▲—▲), with M; (O—O), without M; (x—x), without M for 72 hours before transfer to dishes with M. The values are means ± SEM of five experiments.

adherent cells were cultured in medium consisting of 25 per cent pooled human A serum and 75 per cent RPMI 1640. Experiments were started when the macrophages were 8 days old. The number of macrophages at the start of the experiments was estimated by counting cells in an inverted phase contrast microscope. The cells in 20 visual fields were counted and multiplied by a constant factor estimated from the total number of fields per dish All experiments were performed in medium containing 20 per cent AB serum.

Experiments. The lymphocytes, adjusted to 10^6 cells per ml, were either sedimented on the macrophage layer or kept in the proximity of the macrophages by a double chamber system.

The double chamber system has been described in detail in a preceding article (13). Briefly, polycarbonate membranes (pore size 0.2 μm) were glued to the bottom og glass rings to make membrane chambers. Glass rings with height ½ mm were placed in the bottom of the culture dishes. The double chambers were formed by mounting membrane chambers on these rings Two ml of medium was added to the lower chamber.

The experiments was started by adding 2½ ml of lymphocyte suspension with 50μl reconstituted phytohaemagglutinin (PHA) either directly on to the macrophage layer or to the membrane chamber. In the former type of experiments the ratio of macrophages to lymphocytes was about 1:5. In the latter type, the ratio was about 1:10, because nearly half the macrophages were removed to clear an area for the glass rings.

Assay of DNA, RNA and protein synthesis For registration of DNA synthesis, ^3H thymidine (sp. act. 26 Ci/mmol) was added in a concentration of 0 5 μCi per ml culture medium For registration of RNA and protein synthesis, 1 μCi ^3H uridine (sp act. 46 Ci/mmol) and 1 μCi ^3H leucine (sp. act 1. 0 Ci/mmol), respectively, were added per ml of the culture medium The isotope was present in the culture medium for the last 4 hours before harvesting. The cells were harvested as described in a preceding paper (13).

Statistics. The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

Protein and RNA synthesis in PHA-stimulated lymphocytes sedimented on the macrophage layer. Lymphocyte suspension and PHA were added to culture dishes with or without macrophages. The unstimulated controls were lymphocyte suspensions without mitogen added to dishes without macrophages. The ^3H leucine and ^3H uridine incorporation was registered at different times from the start of the experiments, pulsing with isotope for the last 4 hours before harvesting.

The mitogen had a market effect on protein synthesis, as measures by ^3H leucine incorporation. During the first 24 hours, the rate of ^3H leucine incorporation increased 4-fold in the stimulated lymphocytes as compared to the unstimulated controls (Table 1). During the next 24 hours, there was a further 4-fold increase. During the subsequent 48 hours, the rate of ^3H leucine incorporation increased more abruptly. No increase was registered in the unstimulated controls, making a 50-fold increase in the stimulated as compared to the unstimulated lymphocytes 96 hours after the start of the experiment.

In the stimulated lymphocytes co-cultured with macrophages, there was only a minor increase in ^3H leucine incorporation during the first 24 hours. No further increase was registered during the rest of the period, thus revealing a strong inhibitory effect of

TABLE 1 *Influence of Macrophages on ^3H Leucine Incorporation in PHA-Stimulated Lymphocytes Sedimented on the Macrophage Layer*

Duration of lymphocyte culture	^3H leucine incorporation in c.p m $\times 10^{-3}$			
	Incorporation in lymphocytes			Incorporation in macrophages co-cultured with PHA-stim lymphocytes
	PHA-stimulated		Non-stimulated	
	With M[a]	*Without M*	*Without M*	
24 h	2 3 ± 0 4	4.0 ± 0 5	1.1 ± 0.2	30.1 ± 7.1
48 h	2.5 ± 0.7	14.6 ± 0.5	0.7 ± 0 1	23.0 ± 6.6
72 h	1.9 ± 0.4	34 3 ± 2.8	0.7 ± 0.2	24.3 ± 4 8
96 h	2.6 ± 0.6	50.7 ± 7.0	1.0 ± 0.1	24.1 ± 6 2

Lymphocytes were added to dishes with or without macrophages. The cultures were pulsed with isotope for 4 hours before harvesting. The incorporation in non-adherent cells removed by washing with cold saline was attributed to incorporation in lymphocytes. The incorporation in cells removed by lysing with distilled water was ascribed to incorporation in macrophages. The values are means ± SEM of five experiments.

[a] M = macrophages.

TABLE 2. *Influence of Macrophages on ³H Uridine Incorporation in PHA-Stimulated Lymphocytes Sedimented on the Macrophage Layer*

Duration of lymphocyte culture	³H uridine incorporation in c p m × 10⁻³			
	Incorporation in lymphocytes			Incorporation in macrophages co-cultured
	PHA-stimulated		Non-stimulated	with PHA-stim. lymphocytes
	With M^a)	Without M	Without M	
24 h	18 ± 7	55 ± 15	3 ± 1	127 ± 15
48 h	26 ± 5	211 ± 30	3 ± 1	80 ± 12
72 h	20 ± 8	264 ± 32	2 ± 0	96 ± 15
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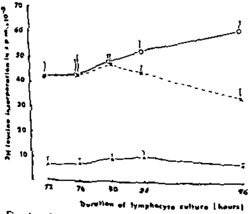


Fig. 1. ³H leucine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M (▲—▲), with M, (O—O), without M, (x—x), without M *for 72 hours before transfer* to dishes with M. The values are means ± SEM of five experiments.

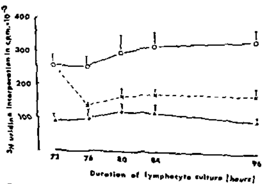


Fig. 2. ³H uridine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M (▲—▲), with M; (O—O), without M; (x—x), without M for 72 hours before transfer to dishes with M. The values are means ± SEM of five experiments.

chambers were transferred from dishes without macrophages to dishes with macrophages. The kinetics of the inhibition were studied by harvesting cultures every 4 hours from the 72nd to the 84th hour after start of the experiment. Some cultures were harvested at 96 hours.

The ^{3}H leucine incorporation in lymphocytes cultured in the proximity of macrophages during the whole experiment was strongly inhibited (Fig. 1). Transfer of lymphocytes to macrophages 72 hours after start of the experiment had no immdiate effect on ^{3}H leucine incorporation. However, 12 hours later there was a small depression and 24 hours later a marked depression in the transferred lymphocytes.

The kinetics of RNA synthesis in lymphocytes influenced by macrophages showed similarities to the macrophage inhibition of DNA synthesis presented in a preceding paper (13). Transfer of lymphocytes to macrophages 72 hours after start of the experiment caused an immediate depression of ^{3}H uridine incorporation to about 54 per cent of the control values (Fig. 2). However, ^{3}H uridine incorporation in lymphocytes cultured in the proximity of macrophages during the whole experiment was depressed to about 38 per cent of controls.

DNA and protein synthesis in PHA-stimulated lymphocytes removed from macrophage cultures. PHA-stimulated lymphocytes were cultured in membrane chambers placed in culture dishes with or without macrophages. Forthy-eight hours after start of the experiment, some of the membrane chambers were transferred from dishes with macrophages to dishes without macrophages. To study the kinetics of DNA synthesis, lymphocyte cultures were harvested every four hour from the 48th to the 60th hour after start of the experiment and subsequently at 72 and 96 hours. The kinetics of protein synthesis were studied by harvesting lymphocyte cultures at 52, 72 and 96 hours.

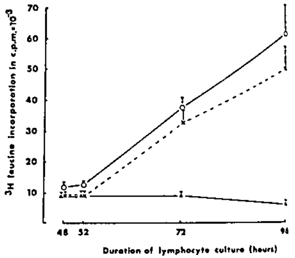


Fig. 4. ^{3}H leucine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M. (▲—▲), with M; (O—O), without M, (x—x), with M for 48 hours before transfer to dishes without M. The values are means ± SEM of five experiments

The results show that macrophage-depressed lymphocytes increase their ^{3}H thymidine incorporation abruptly during the first 12 hours after transfer to dishes without macrophages (Fig. 3). Eight hours after transfer, the ^{3}H thymidine incorporation was larger in the transferred lymphocytes than in the control lymphocytes cultured without macrophages during the whole experiment. However, the difference was not significant ($p > 0.05$) Twelve hours and 24 hours after transfer, the ^{3}H thymidine incorporation was significantly larger in the transferred lymphocytes than in the controls ($p < 0.05$) After another 24 hours the difference had disappeared

The inhibition of ^{3}H leucine incorporation in lymphocytes cultured for 48 hours in the proximity of macrophages was not relieved 4 hours after transfer of lymphocytes to dishes without macrophages (Fig. 4). At subsequent registrations, there was a considerable increase in ^{3}H leucine incorporation in transferred lymphocytes, but not to the level of controls cultured without macrophages during the whole experiment.

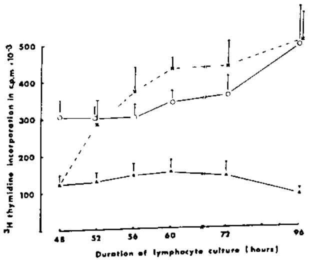


Fig. 3. ^{3}H thymidine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M. (▲—▲), with M; (O—O), without M, (x—x), with M for 48 hours before transfer to dishes without M. The values are means ± SEM of six experiments.

DISCUSSION

^3H leucine incorporation is a reasonably accurate index of the rate of protein synthesis (6). However, ^3H uridine incorporation is not a true reflection of the RNA synthesis in stimulated as compared to unstimulated lymphocytes, because the rate limiting step in ^3H uridine incorporation in unstimulated lymphocytes might be the transport of precursor through cell membranes (3, 10). ^3H uridine incorporation is a more accurate index for registration of depressed RNA synthesis in PHA-stimulated lymphocytes.

Both ^3H leucine and ^3H uridine incorporation was inhibited when PHA-stimulated lymphocytes were cultured in contact with macrophages or in the proximity of macrophages for 96 hours. This is in accordance with preceding results (13) showing inhibition of DNA synthesis and proliferation.

A possible explanation of the inhibition of ^3H leucine and ^3H uridine incorporation in stimulated lymphocytes sedimented on the macrophage layer might be isotope depletion of the medium by the macrophages However, this explanation can be excluded for the following reasons The maximum isotope incorporation registered in the stimulated lymphocytes cultured alone exceeded the sum of the incorporation in lymphocytes and macrophages cultured together, thus showing that there was enough isotope available Furthermore, if lymphocytes and macrophages were competing for isotope, the reduced isotope incorporation in lymphocytes would be expected to increase with culture time as long as the incorporation in macrophages did not increase No significant increase was observed either in macrophages or in co-cultured lymphocytes In the membrane chamber experiments, isotope depletion was even less likely because a larger volume of medium with isotope was available

Waldman & Gottlieb (16) found a moderate inhibition of ^3H uridine and ^{14}C amino acid incorporation in non-stimulated lymphocytes co-cultured with rat peritoneal macrophages The ratio of macrophages to lymphocytes was 1 5 In our experiments, human macrophages co-cultured with PHA-stimulated lymphocytes in a similar ratio caused a very strong inhibition of ^3H uridine and ^3H leucine incorporation The inhibition was strong, even though the cell populations were separated by a membrane concomitant with a reduction in the macrophage/lymphocyte ratio to 1·10 This indicates that both RNA and protein synthesis is inhibited by a soluble factor(s) released by macrophages

The kinetics showed that DNA and RNA synthesis in stimulated lymphocytes were readily inhibited in the proximity of macrophages This depression may be a result of a blocking effect on nucleic acid synthesis affecting both replication and transcription. The delayed inhibitory effect on protein synthesis may reflect that mRNA is available in sufficient amounts for at least 8 hours after depression of DNA and RNA synthesis. This is in accordance with the stability of mRNA in mammalian cells (5, 12).

DNA synthesis in lymphocytes removed from macrophages 48 hours after start of the experiment readily reached the level of DNA synthesis in control lymphocytes, thus indicating a cytostatic effect. Twelve and 24 hours after removal, the DNA synthesis in these lymphocytes was significantly higher than in control lymphocytes. This might be due to synchronization of the DNA synthesis because of accumulation of lymphocytes in G_0 or G_1 phase during macrophage depression. Control lymphocytes finish their first S phase and mitosis in 48 hours (2, 11) Protein synthesis in lymphocytes removed from macrophages showed the same increase as protein synthesis in control lymphocytes, though it did not reach the level of the controls Since the cells synthesize protein during all phases of the cycle, this observation probably reflects the inhibited proliferation during exposure to macrophages with fewer lymphocytes in the cultures removed from macrophages than in the controls

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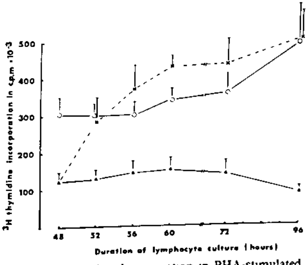


Fig 3 ^{3}H thymidine incorporation in PHA-stimulated lymphocytes cultured in membrane chambers mounted on ½ mm high rings placed in culture dishes with or without M. (▲—▲), with M; (O—O), without M, (x—x), with M for 48 hours before transfer to dishes without M. The values are means ± SEM of six experiments

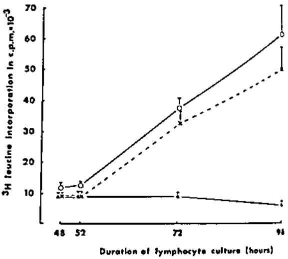


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ISOLATION OF ENZYMATICALLY DERIVED FRAGMENTS OF GUINEA PIG IgG AND AN EXAMINATION OF THEIR REACTIVITY AGAINST STAPHYLOCOCCAL PROTEIN A

C ENDRESEN and A GROV

The Gade Institute, Department of Microbiology, University of Bergen, Bergen, Norway

Endresen, C & Grov, A Isolation of enzymatically derived fragments of guinea pig IgG and an examination of their reactivity against staphylococcal protein A Acta path microbiol scand Sect. C, 86 193–198, 1978

Papain digest of guinea pig IgG_2 was separated by ion-exchange chromatography into five fractions, one containing mainly Fab and four Fc fractions Covalently linked Fc (cFc) and non-covalently linked Fc fragments (nFc) were present, both with a molecular weight (Mw) of about 56,000 nFc was split into half fragments by gel filtration under dissociating condition (Mw 25,000 to 27,000) An incomplete Fc fragment (iFc) was also isolated (Mw 36,000), which consisted of a Fc chain (Mw 26,000) non-covalently linked to a piece of another chain These preparations all reacted with protein A In addition, the Fc' fragment (Mw 22,000), which is the C-terminus of the Fc fragment, was isolated This fragment did not react with protein A The cFc was treated with acid and then digested with trypsin at pH 7 8 for 45 s The digest were separated into four fractions (I–IV) by gel filtration on Sephadex G-100 Fraction I was indistinguishable from intact Fc, fraction II contained an incomplete covalent Fc fragment (Mw 34,000 to 38,000) Both these fragments reacted with protein A Fraction IV consisted of fragments belonging to the C-terminus, and was protein A non-reactive. Fraction III was shown to contain a mixture of fragments belonging to fractions II and IV

Key words Fc subfragments, guinea pig IgG, protein A interaction

C Endresen, Mikrobiologisk avdeling, MFH-bygget, N-5016 Haukeland sykehus, Norway.

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Normal IgG from most mammalian species react with staphylococcal protein A, the reaction site being localized to the Fc part of the γ-globulin (9, 10, 11, 12, 16) The nature of this Fc-protein A interaction is, however, poorly understood. Previous examinations of isolated Fc subfragments of human IgG have shown that only those fragments having an intact Fc chain are reactive to protein A (5, 6) The purpose of this study was to examine the protein A reactivity to guinea pig IgG_1 and IgG_2, reacting with protein A through the Fc region (8), in order to find out if there is an analogous reaction to that of human IgG. Various Fc fragments produced by papain digestion of IgG_2 (1, 15) and subfragments of Fc derived by trypsin digestion were characterized and included in the experiments.

MATERIALS AND METHODS

Purification of guinea pig IgG_2 and IgG_1 from pooled normal sera was performed by ammoniumsulphate precipitation and DEAE ion exchange chromatography as described in (14) The isolated γ-globulin pools were further gel filtered through columns of Sephadex G-200 (3.2 × 92 cm) equilibrated with 0 1 M Tris-HCl, 0 15 M NaCl, 2 mM EDTA-Na_2 and 0 02 per cent NaN_3, pH 7 8, to obtain monomeric γ-globulin fractions The protein solutions were concentrated by pressure dialysis to concentrations of 15 mg/ml and stored at −22° C

Papain digestion of IgG_2 was performed as described in (21) The Fab and Fc products from papain digestion were isolated by DEAE-cellulose chromatography (Whatman DE-32, England) using 50 mM Tris-HCl, pH 8 2 (200 ml), and a linear salt concentration gradient (0–0 4 M NaCl) as described in (15) The isolated Fc fragments were further separated on Sephadex G-100

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material contained the same antigenic determinants as those of peak A and B (Fig 2a). Peak D contained apparently only one fragment Gel filtration under neutral conditions showed one peak with an apparent molecular weight of 22,000, whereas gel filtration under dissociating conditions as well as SDS electrophoresis (Fig 1) gave a molecular weight about 10,000. This fragment contained less antigenic determinants than fragments A, B, and C, and was more anodical than the others (Fig 2b)

The elution profile from Sephadex gel filtration of trypsin-digested Fc fragments is illustrated in Fig. 3, and the material was divided into four fractions (I-IV) as indicated Apparently fraction I contained almost undigested Fc fragments (Fig. 4) Fraction II contained two fragments slightly different in size according to the SDS electrophoresis (Fig. 4), but were antigenically indistinguishable according to double diffusion and immunoelectrophoresis analysis (Fig 5) SDS-polyacrylamide gel electrophoresis of reduced and alkylated material (Fig. 4) indicated that both fragments consisted of one intact Fc chain covalently bound to a segment of the other chain Fraction IV appeared to be identical to the papain-derived D fragment (Fc') concerning size and antigenic properties (Figs 4 and 5), whereas fraction III contained a mixture of one of the fragments found in II and the fragment of IV. All fragments precipitated with the anti-Fc'γ_{2}, indicating that no fragments containing only the C_H2 domain were obtained

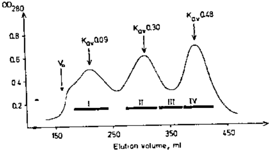


Fig 3 Gel filtration on Sephadex G-100 of a tryptic digest of acid-treated Fc from guinea pig IgG_2 The column (3 2 × 78 cm, Vo = 169 ml) was equilibrated and eluted with 1 0 M acetic acid containing 25 mM NaCl (elution rate 18 ml per h).

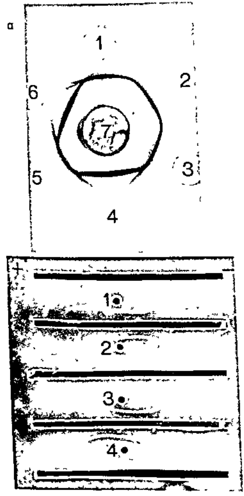


Fig 2 a Double diffusion in agar gel The wells were filled with A (1) B (2 and 5), C (3), D (4 and 6), and the center well with rabbit anti-guinea pig $Fc\gamma_2$ serum b Immunoelectrophoresis of A (1), B (2), C (3), D (4) Troughs no 2 and 4 were filled with rabbit anti-guinea pig $Fc\gamma_2$ specific serum, the other troughs were filled with rabbit anti-guinea pig $F(ab')_2$ serum

All fragments isolated were tested for protein A reactivity as previously described (5, 6). Samples (2 mg protein) were passed through a Sepharose-protein A column with a binding capacity of 5.4 mg purified Fc The results are listed in Table 1. Only

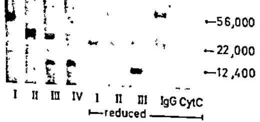


Fig 4 SDS polyacrylamide gel electrophoresis of fractions I–IV both non-reduced and reduced with 5 per cent mercaptoethanol IgG and cytochrome C (both reduced) were run as marker proteins

(3.2 × 90 cm) columns equilibrated in 0.1 M Tris-HCl buffer pH 7.8, 0.2 M NaCl, 2 mM EDTA-Na_2 and 0.02 per cent NaN_3. The fragments were then freed from traces of Fab contamination by passage through a column (1 × 15 cm) of Sepharose 4 B containing covalently linked IgG antibodies specific for guinea pig Fab. The specific rabbit antibodies were coupled to Sepharose 4 B according to *Porath et al.* (23). Further separation of the Fc fragments were performed by filtration on Sephadex G-100 and G-200 columns in 5 M guanidine HCl-1 M HAc.

Trypsin derived Fc sub-fragments were prepared by the procedure of *Ellerson et al.* (4). Purified Fc (20 mg/ml) in 0.15 M NaCl with 10 mM Tris-HCl buffer, pH 7.8, was exposed to acid (pH 2.5) for 5 min at 25° C. Trypsin (Type XI, DCC treated, Sigma, USA) solution was added (the enzyme to substrate ratio was 1:50), and the pH was rapidly readjusted to 7.8 with 2 M Tris After 45 s at 25° C the trypsin was inactivated using an equal weight of soy bean trypsin inhibitor (Sigma, Type I-s) The digest was then gel filtered on a Sephadex G-100 column (3.2 × 78 cm) in 1 N HAc containing 25 mM NaCl.

Antisera to guinea pig IgG_2, $Fc\gamma_2$ and $Fc'\gamma_2$ were obtained from rabbits given two intramuscular injections of antigen in complete Freund's adjuvant (Difco, USA), 2 weeks apart. Two booster injections (2 mg in each) were given subcutaneously, 8 and 10 weeks after the first injection. Blood was drawn from the marginal ear vein 6 to 7 days after each booster injection.

Polyacrylamide gel electrophoresis with SDS was performed according to *Fairbanks et al* (7) using 0.1 per cent SDS in the gel The protein materials were prepared for electrophoresis by dialysis against 1 per cent SDS, 10 per cent sucrose, 10 mM Tris-HCl (pH 6.8), 1 mM EDTA, 0 8 M urea and 4 per cent mercaptoethanol, and heated to 100° C for 60 s.

Immunoelectrophoresis was performed using special Agar Noble (Difco) in barbitone buffer (I, 0 025; pH 8 6) and a voltage of 6–8 V/cm.

The apparent molecular weight of the Fc subfragments was determined by the method of *Andrews* (2) using the K_{av} values of the gel filtered proteins (13) The peptides were reduced and alkylated (20), then gel filtered through Sephadex G-200 (1 6 × 93 cm) and Sephadex G-100 (1 6 × 89 cm) columns in 5 M guanidine HCl-1 M HAc. Dextran Blue (Pharmacia, Sweden), and DNP-alanine (Nutritional Biochem Co., USA), were used to determine the void volume and total volume respectively. Heavy chains (γ_2), L-chains and cytochrome C (Sigma, USA) were employed as standard marker proteins

Double diffusion in agar was carried out as described by *Ouchterlony* (22) in 1 per cent Agar Noble (Difco) in barbitone buffer (I, 0 025; pH 8.6)

The concentrations of proteins were performed by ultrafiltrations using Amicon cells and UM-2 filters (Diaflo, The Netherlands), and the protein concentration determined as described in (17)

RESULTS

The separation by DEAE ion-exchange chromatography of the papain derived fragments of guinea pig IgG_2 is shown in Fig. 1. The Fc fragments were eluted in four peaks (A, B, C, and D). The fractions under each peak were bulked as indicated, freed from traces of Fab fragments on the immunoadsorbent column, and further examined by gel

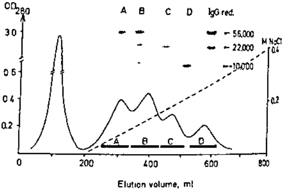


Fig 1. DEAE ion-exchange chromatography of papain digest of guinea pig IgG_2. The column (2.5 × 42 cm) was equilibrated with 50 mM Tris-HCl pH 8 0 Undigested IgG_2 and Fab fragments were eluted off the column with 200 ml of buffer, and the Fc material was released using a linear gradient of 300 ml buffer in the mixing chamber and 300 ml buffer with 0 4 M NaCl in the reservoir chamber. Elution rate· 40 ml per h The fractions A, B, C, and D were all bulked as indicated. The SDS-polyacrylamide gel electrophoresis of the main fraction of each peak is illustrated Reduced IgG was run as a marker of molecular weights

filtration on Sephadex G-200 and G-100 columns, both with and without guanidine HCl-acetic acid as a dissociating agent. Fraction A and B both contained two different fragments Gel filtration under non-dissociating conditions gave only one peak with an apparent molecular weight of 56,000, whereas 2 peaks with apparent molecular weights of 56,000 and 25,000 to 27,000, were revealed under dissociating conditions. The presence of these fragments in A and B was also verified by SDS polyacrylamide electrophoresis (Fig 1). The apparent molecular weights were estimated by the same method. Double diffusion experiments with A and B gave one line of complete identity against anti-IgG_2 (Fig. 2a). In addition to smaller amounts (approx. 10 per cent) of the same fragments as found in A and B, peak C contained a fragment with an apparent molecular weight of about 36,000. Gel filtration under dissociating conditions revealed two peptide chains with apparent molecular weights of about 26,000 and 10,000. This was also confirmed by SDS electrophoresis (Fig. 1). The peak C

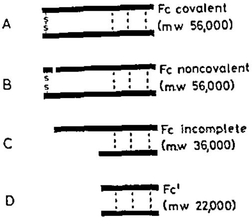


Fig 6 Proposed structure and molecular weights of papain derived Fc subfragments of guinea pig IgG_2 as described by *Alexander et al* (1)

indicates that trypsin primarily splits between the C_H2 and C_H3 domains, and that free C_H2 peptides are further rapidly degraded by the enzyme. On the other hand, C_H3 homology regions in the form of pFc', tFc' and Fc' fragments are all relatively resistent to further digestion.

Protein A reactive fragments were found in the tryptic derived fractions I and II, whereas no such activity was observed in the fraction IV fragments

Non-reactive fragments were also present in fraction I and II These fragments were both chemically and serologically indistinguishable from the protein A reactive fragments of the same fraction This was also the case for corresponding fragments of human Fc (5, 6) Probably, small differences in the amino acid sequence or in the tertiary structure of Fc fragments may affect their reactivity with protein A

Protein A reactive papain derived fragments were isolated from fraction A and B. About 80 per cent of all peptides in these fractions were fixed to the

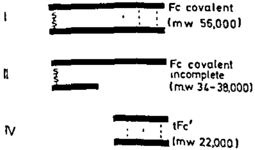


Fig 7 Proposed structure and molecular weights of trypsin derived Fc subfragments

Sepharose-protein A column. Only slight reactivity (about 15 to 20 per cent) was also found in fraction C, containing about 90 per cent Fc incomplete fragments, indicating that the latter fragments barely reacted with protein A. This is in accord with the previous conclusions (5, 6) that one intact Fc-chain is necessary for protein A reactivity. C_H2 and C_H3 fragments of guinea pig IgG prepared by cyanogen-bromide treatment according to (3) were also non-reactive to protein A (*Endresen*, unpublished data) The non-reactive Fc incomplete fragments apparently had lost too much of their N-terminal structure. The necessity of the N-terminus can be important on stabilizing the cis-interaction between C_H2 and C_H3 (18, 19), an interaction which might be of great importance to the protein A-Fc interaction as well

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the papain-derived fragment D and the trypsin-derived fragment IV were completely non-reactive to protein A.

a

b

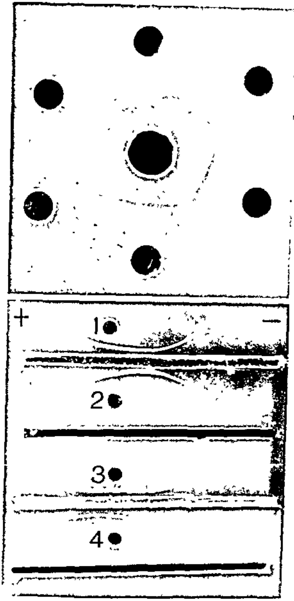


Fig. 5 a. Double diffusion in agar gel. The wells were filled with I (1), II (2), III (3), IV (4), Fc' (5), and rabbit anti-guinea pig Fcγ_2 serum in the center well b. Immunoelectrophoresis of I (1), II (2), III (3), IV (4). Antisera were placed as described for Fig. 2b.

TABLE 1 *Protein A Reactivity of Various Fc Fragments and Subfragments as Estimated on a Sepharose-Protein A Column*

Fractions	Protein A reactivity per cent
A	80
B	80
C	15–20
D	0
I	75
II	65
IV	0

A–D Papain derived fragments
I–IV. Trypsin derived fragments

DISCUSSION

The Fc fragments of IgG_2 derived by papain digestion are apparently of four different types Fractions A and B contained one fragment which both chemically and antigenically was equal to intact Fc. In addition, both fractions contained another fragment of the same size as intact Fc, but which under dissociating conditions split into two sub-fragments (Mw 25,000 to 27,000) which were indistinguishable both by gel filtration and by electrophoresis. These findings are in accord with those of *Alexander et al.* (1) who called these fragments covalently linked Fc (cFc) and non-covalently linked Fc (nFc), respectively (Fig. 6) The fragment found in fraction C with an apparent molecular weight of 36,000 is likely to be a Fc incomplete fragment (iFc) (Fig. 6). This fragment contained one complete Fc-chain with a non-covalent bound piece of another chain. This piece may be of variable size (Mw 10,000 to 14,000). Fraction D was composed of two non-covalently bound sub-fragments of similar size. According to the serological tests, this fragment probably belongs to the C-terminus of Fc and is likely to be the so-called Fc' fragment (Fig. 6). No free fragment of the C_H2 domain was observed, thus confirming all previous observations by *Alexander et al* (1). The heterogenious pattern of fragments after papain hydrolysis of guinea pig IgG_2 was different from similar hydrolysis of human and rabbit IgG, both giving a higher yield of Fc covalent fragments.

The proposed structure of fragments after tryptic digestion of acid treated Fc is shown in Fig. 7. Fraction I contained undigested Fc. Fraction II contained fragments with one complete Fc chain covalently linked to a piece of variable size from another chain, and fraction IV contained non-covalently linked fragments from the C-terminus (tFc' fragments). The number of fragments obtained with guinea pig Fc was more limited than when human Fc was used (5, 6) C_H2 domain fragments could not be isolated from guinea pig, probably because of a more rapid degradation than for corresponding human fragments. Attempts to recover free C_H2 peptides using a shorter digestion time than 45 s and an enzyme. substrate ratio of 1.100 was not successful. Unlike other domains, the C_H2 homology region was found very susceptible to enzyme attack. Most probably, the C_H2 region is progressively degraded Fragments isolated from peak II contained pieces of the N-terminal half covalently bound to the other intact Fc chain. This

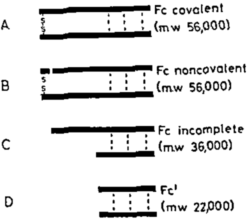


Fig 6 Proposed structure and molecular weights of papain derived Fc subfragments of guinea pig IgG_2 as described by *Alexander et al* (1)

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Protein A reactive papain derived fragments were isolated from fraction A and B About 80 per cent of all peptides in these fractions were fixed to the

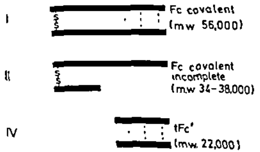


Fig 7 Proposed structure and molecular weights of trypsin derived Fc subfragments

Sepharose-protein A column. Only slight reactivity (about 15 to 20 per cent) was also found in fraction C, containing about 90 per cent Fc incomplete fragments, indicating that the latter fragments barely reacted with protein A. This is in accord with the previous conclusions (5, 6) that one intact Fc-chain is necessary for protein A reactivity. C_H2 and C_H3 fragments of guinea pig IgG prepared by cyanogen-bromide treatment according to (3) were also non-reactive to protein A (*Endresen*, unpublished data). The non-reactive Fc incomplete fragments apparently had lost too much of their N-terminal structure. The necessity of the N-terminus can be important on stabilizing the cis-interaction between C_H2 and C_H3 (18, 19), an interaction which might be of great importance to the protein A-Fc interaction as well

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Acta path microbiol. scand Sect. C, *86* 199–201, 1978

BRIEF REPORT

IMMUNOSUPPRESSIVE EFFECT OF HUMAN PREGNANCY ZONE PROTEIN ON H-2 INCOMPATIBLE MOUSE HEART ALLOGRAFTS

P Svendsen[1], T. Stigbrand[2], B Teisner[3], J Folkersen[3], M.-G Damber[4], B von Schoultz[4], E. Kemp[5] and S-E Svehag[3]

[1] Laboratory Animal Unit, [3] Institute of Medical Microbiology and [5] Institute of Internal Medicine, Odense University, Odense, Denmark. [4] Department of Obstetrics and Gynaecology and [2] Department of Physiological Chemistry, Umeå University, Umeå, Sweden

Svendsen, P, Stigbrand, T, Teisner, B, Folkersen, J, Damber, M.-G., von Schoultz, B, Kemp, E. & Svehag, S-E Immunosuppressive effect of human pregnancy zone protein on H-2 incompatible mouse heart allografts Acta path. microbiol scand Sect C, *86* 199–201, 1978

Human pregnancy zone protein (PZP) given intraveneously, significantly ($p<0.001$) prolonged the survival of heterotopic A/J heart transplants in A CA recipients, compared to untreated control recipients, recipients treated with phosphate buffered saline and recipients treated with a control preparation from male serum The results support the hypothesis that PZP protein exerts an unspecific immunosuppressive effect in vivo

Key words Pregnancy zone protein, immunosuppression; mouse heart allografts.

P Svendsen, Laboratory Animal Unit, Odense University, DK-5000 Odense, Denmark

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The immunological paradox of pregnancy has attracted much interest since it was first suggested that pregnancy could be compared to organ transplantation from an immunological point of view (6) Several theories have been advanced in attempts to explain the survival of the foetal allograft (5) Pregnancy zone protein (PZP) has been suggested to be one of the unspecific immunosuppressive factors operating in pregnancy because of the marked elevated serum concentration in pregnant women (1, 2, 8, 13, 16) Immunosuppressive properties have been demonstrated in in vivo tests (3 7, 9, 10, 12, 15) The biological importance of PZP, however, is largely unknown, although some selective affinity for monocyte- and lymphocyte subpopulations has been suggested (11)

In the present study the immunosuppressive effect of human PZP was examined in an in vivo model making use of heterotopic transplantation of strongly histoincompatible mouse heart allografts

Materials and Methods

Purification of PZP protein Serum from pregnant women in the third trimester was diluted with 1 25 volumes of 0 03 M NaK-phosphate buffer, pH 7.2. Aetacridin-lactate was added to a final concentration of 0 4% w/v and pH adjusted to 6 0 The mixture was incubated for 30 minutes and the precipitate pelleted at 2500 *g* for 15 minutes The precipitate was redissolved in twice the original serum volume of 0 83 M NaCl and centrifuged at 1500 *g* for 15 minutes The supernatant was dialysed against 0 03 M NaK-phosphate buffer, pH 7 2 and gelfiltered on Sepharose 6 BCL. The fractions were tested by fused rocket immunoelectrophoresis using a monospecific rabbit anti-human PZP serum PZP containing fractions were concentrated, dialysed against phosphate buffered saline (PBS) and sterile filtered

Production of male serum derived control preparation (MCP) Male serum without detectable levels of PZP (< 0 2 µg/ml) went through the same purification procedure as the PZP-containing pregnancy serum. As PZP

various species origin. Acta path. microbiol. scand. Sect. B, *78:* 106–111, 1970.

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Discussion

Pregnancy zone protein has been suggested to exert an unspecific immunosuppressive effect during pregnancy. This hypothesis is based on the observed immunosuppressive effect of PZP in in vitro tests But the conclusion that can be drawn from assessing the effect of PZP on lymphoid cells in in vitro tests are limited. Therefore it was decided to examine the possible immunosuppressive effect of human PZP in a heterologous *in vivo* transplantation system Optimal conditions for the in vivo testing of PZP would require the use of a primate transplantation model and the homologous PZP analogue. Technical and economical reasons have precluded the use of this in vivo model Analogues to human PZP have been detected in apes, monkeys, dogs, cows and sheep by immunological techniques (17) Pregnancy associated mouse proteins with α_1, α_2 and β-mobility have been described (4), but no antigenic cross-reactivity to human PZP was observed

The observation that elimination of PZP from mouse serum is faster after the first injection than after repeated injections suggests a saturation of sites with specific or non-specific affinity for PZP The family of elimination curves (Fig 1) indicates no immunological elimination of the protein in the period studied

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Previous investigations have demonstrated an inhibitory effect of PZP on T-lymphocyte functions in vitro The results of the present investigation are in accordance with the hypothesis that the PZP also affects the cell mediated immune response in vivo

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constituted about 60% of the protein content in the test preparation, human serum albumin from male serum was added to the MCP *preparation to give the same total* protein content in both preparations.

PZP determination. PZP concentration was determined by rocket immunoelectrophoresis (18) using a monospecific rabbit antiserum to human PZP. The electrophoresis was performed with 0.028 M barbital buffer at pH 8.6 and 1 V/cm gel for 18 hours

Transplantation model. Hearts of infant A/J BOMf (H-2a) mice, less than 24 hours old, were grafted bilaterally in a 5 mm deep subcutaneous pouch in the dorsal pinna of adult male A.CA (H-2f) mice (14) The action potentials of the transplanted heart muscle were recorded daily beginning at day seven following surgery. Needle electrodes were inserted on either side of the graft and in the tail, and potentials were amplified on an ECG amplifier EAP 205 and recorded on a trendscope MTS 216. Termination of electrical activity indicated rejection of the graft. Mice were anaesthetized during surgery and ECG recording with 0.3 ml Epontol® injected intraperitoneally.

The A.CA recipients (in total 127) were divided into five groups, one of which served as an untreated control group. Group two was injected 84, 96, 108, 132 and 156 hours after surgery with 0.5 ml PBS. Groups three and four were injected at similar intervals with 0 5 ml MCP or PZP (4 mg/ml). To study the influence of injection time of PZP in relation to surgery, group five was injected with 0.5 ml PZP solution (4 mg/ml) at 0, 12, 24, 48 and72 hours after surgery All injections were given intraveneously in the tail vein

Elimination of PZP protein Elimination of PZP from the serum was studied in two experiments. In the first, two mice were injected with 1 mg PZP (0.5 ml) intraveneously Blood samples were drawn from the periorbital plexus 5, 30, 60, 120 and 360 minutes after the injection

In the second experiment elimination of PZP was examined in five groups with four animals in each group The first group received one dose of 550 µg PZP (0 5 ml), the second group two doses etc. with intervals similar to those used in transplanted mice Blood samples were taken 1, 2, 4, 24, 48 and 72 hours after each injection

Results

Elimination of PZP from mice Intraveneous injection of 1 mg PZP resulted in a blood concentration of approximately 650 µg/ml five minutes after the injection. The elimination curve demonstrated a reduction to half that concentration in 30 minutes, and a further reduction to one quarter of the initial concentration in two hours.

In the long term elimination experiment (Fig 1), the first injection resulted in a comparable initial concentration and elimination rate of PZP as in the short term experiment. Repeated injections with 12 to 24 hour intervals increased the initial PZP concentration and reduced the rate of elimination from the blood The five injections given at intervals similar to those in the transplantation experiment could maintain a minimum blood PZP level of 100 µg/ml for three to four days

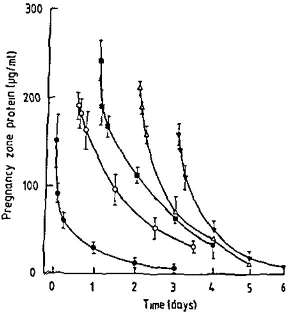


Fig 1. Elimination of human pregnancy zone protein from serum of five groups of mice given one or repeated doses of 550 µg PZP. ● – ● one injection, ○ – ○ two injections, ■ – ■ three injections △ – △ four injections and ▲ – ▲ five injections. The first sample was taken one hour after each injection. mean ± 1 SD for four animals in each group

Transplantation experiments Comparison between mean graft survival time for untreated and PBS treated mice showed no significant difference ($p > 0.1$) (Table 1) Injection of MCP resulted in a significantly prolonged graft survival time compared to untreated ($p < 0.001$) and PBS treated mice ($p < 0.02$) Both groups treated with PZP (5 × 0.5 ml with 4 mg/ml) had significantly longer graft survival times than untreated mice ($p < 0.001$), PBS treated mice ($p < 0.001$) and MCP treated mice ($p < 0.001$) The time of application of PZP to surgery had no signigicant influence on the graft survival time ($p > 0.7$).

TABLE 1. *Graft Survival Times for Heterotopic A/J BOMf (H-2a) Heart Transplants in A CA (H-2f) Recipients Treated with 10 mg Human Pregnancy Zone Protein (PZP), 10 mg Male Serum Derived Control Preparation (MCP) and Phosphate Buffered Saline (PBS)*

Treatment	No of recipients	Graft survival time (mean)	S D
No treatment	23	11 0	1 7
PBS day 3½ to 6½	29	11 9	1 6
MCP day 3½ to 6½	34	13 1	2 7
PZP day 3½ to 6½	27	15 7	2 7
PZP day 0 to 3½	14	15.4	1.5

Discussion

Pregnancy zone protein has been suggested to exert an unspecific immunosuppressive effect during pregnancy. This hypothesis is based on the observed immunosuppressive effect of PZP in in vitro tests But the conclusion that can be drawn from assessing the effect of PZP on lymphoid cells in in vitro tests are limited Therefore it was decided to examine the possible immunosuppressive effect of human PZP in a heterologous in vivo transplantation system Optimal conditions for the in vivo testing of PZP would require the use of a primate transplantation model and the homologous PZP analogue Technical and economical reasons have precluded the use of this in vivo model. Analogues to human PZP have been detected in apes, monkeys, dogs, cows and sheep by immunological techniques (17). Pregnancy associated mouse proteins with α_1, α_2 and β-mobility have been described (4), but no antigenic cross-reactivity to human PZP was observed

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BRIEF REPORT

PRODUCTION OF AN OCHRATOXIN A ANTIGEN WITH HIGH HAPTEN/CARRIER MOLAR RATIO

Helle Worsaae

Department of Forensic and State Veterinary Medicine, Royal Veterinary and Agricultural University, Copenhagen, Denmark

Worsaae, H Production of an Ochratoxin A antigen with high hapten/carrier molar ratio Acta path microbiol scand Sect. C, *86* 203–204, 1978

A mixed anhydride method is described for coupling ochratoxin A to bovine serum albumin. The obtained molar ratio of hapten/carrier in the coupling product was 20 Antibodies against ochratoxin A was obtained from rabbits after immunization with the ochratoxin A-bovine serum albumin conjugate.

Key words Ochratoxin A, hapten, hapten/carrier conjugates

Helle Worsaae, Royal Veterinary and Agricultural University, Department of Forensic and State Veterinary Medicine, 13, Bulowsvej, DK-1870 Copenhagen V, Denmark

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The potent mycotoxin ochratoxin A (OA) has been shown to be causally associated with toxic nephropathy in farm animals in Denmark (*Krogh et al* 1973, *Elling et al* 1975) To quantitate OA in tissue or blood a specific and highly sensitive assay is needed

Radioimmunoassays (RIA) for OA have been developed using antibodies synthesized against OA coupled to bovine serum albumin (BSA) via the action of a derivative of carbodiimide (*Aalund et al* 1975, *Chu et al* 1976) *Chu et al* (1976) achieved an OA/BSA molar ratio of 8–10 in the coupling product.

It is a general experience that a hapten-protein molar ratio of less than 10 is associated with the production of low affinity antibodies obtained in only low titres (*Abraham* 1974) However when the molar ratio is 20 or more it is possible to induce the synthesis of antibodies with high affinity and frequently also to obtain high titres This is essential since the sensitivity of RIA is directly proportional to the affinity of the antibodies (*Abraham* 1974)

In this paper a procedure is described for coupling OA to BSA using a modification of the mixed anhydride method (*Erlanger et al* 1957) to obtain a hapten/carrier molar ratio of 20 in the coupling product.

Materials and Methods

Crystalline OA prepared from cultures of *Aspergillus ochraceus* (*Krogh et al* 1976) was readily soluble in tetrahydrofurane (THF)

Just prior to use solvent (THF) and reagents (tri – n – butylamine, isobutylchlorocarbonate) employed in the carboxylic acid anhydride reaction had been distilled and rendered anhydrous over calcium chloride.

The OA-BSA coupling included the following steps

1. 40 3 mg (0 1 m mol) OA was dissolved in 1 ml THF A separate aliquot of 1 ml THF followed through the procedure as a control
2. 25 µl (0 1 m mol) tri – n – butylamine was added After cooling to 10° C, 15 µl (0 1 m mol) isobutylchlorocarbonate was added
3. The reaction was allowed to proceed for 30 min at 10° C with frequent mixing
4. 167 mg (0 002 m mol) BSA was dissolved in 8 7 ml ice-cold water/THF (v/v 25 35) containing 170 µl 1N sodium hydroxide solution
5. The carboxylic acid anhydride solution was added to the BSA solution under stirring which was continued at 4° C for 4 h. After one hour, pH was adjusted to 9 0

6. The mixture was dialyzed against tap water overnight.
7. BSA was precipitated at pH 4.5 overnight at 4° C using 1N hydrochloric acid.
8. Following centrifugation at 3000 × g at 4° C for 30 min, the precipitate was reconstituted in 2 ml Tris buffer, 0.05 M, pH 8.5.
9. The solution was subjected to gel filtration on a 2.5 × 40 cm column of sephadex G-100 using Tris buffer, 0.05 M, pH 8 5 as the eluent.
10. OA had an absorption maximum at 380 nm in Tris buffer, 0.05 M, pH 8.5, and the extinction coefficient was determined to be 8.0 × 10^3 M^{-1} cm^{-1} at the above wave length. The absorbance of the fractions after gel filtration was measured at 380 nm and 1 cm light path. Ninetysix per cent of the total amount of eluted OA appeared in the V_e/V_t interval 0.21 to 0 38 corresponding to the elution *site for the OA-BSA coupling product Four per cent of the OA; i.e. residual free OA, appeared in the* V_e/V_t interval 0.81 to 1.0. V_e and V_t is the elution volume and the volume of the gel bed, respectively.
11. Fractions representing the V_e/V_t interval 0.23 to 0.34 were pooled to form the OA-BSA fraction.
12. OA-BSA and control-BSA were dialyzed against deionized water and lyophilized.
13. The dried material was dissolved in Tris buffer, 0.05 M, pH 8.5 to a concentration of 0.2 mg/ml.
14. The difference in light absorbance at 380 nm and 1 cm light path between the OA-BSA solution (0.2 mg/ml) and the control-BSA solution (0.2 mg/ml) was found to be 0.41. Hence, the concentration of OA in the solution of OA-BSA was 0.051 mM and the concentration of BSA 0.0026 mM (Mol. wt. of BSA = 70,000), giving an OA/BSA molar ratio of 20 in the coupling product.

Results and Discussion

The coupling product has been used to immunize rabbits for the production of anti OA antibodies employed in studies on localization of OA in the kidney of pigs and rats by an indirect immunofluorescence technique (*Elling* 1977). The antiserum contained anti-BSA antibodies, but retained its anti-OA activity after absorption with BSA. The concentration and specificity of the OA antibodies were sufficiently high to allow the immunofluorescent tracing (*Elling* 1977)

In experimental as well as in clinical work there is a demand for specific OA tests with sufficient sensitivity to allow the analysis to be performed on small aliquots of sera. The above OA-BSA antigen would seem to meet the primary requirement for production of OA antibodies suitable for perfection of a RIA for OA in serum

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Acta path microbiol. scand Sect C, *86* 205–209, 1978.

KINETICS OF LOCOMOTION OF HUMAN GRANULOCYTE POPULATIONS

AKSEL SCHREINER and DAG VAULA

Medical Department B and Institute of Hygiene and Social Medicine, Faculty of Medicine, University of Bergen, N-5016 Haukeland Sykehus, Bergen, Norway

Schreiner, A & Vaula, D Kinetics of locomotion of human granulocyte populations. Acta path. microbiol scand. Sect C, *86* 205–209, 1978

The locomotor kinetics of human granulocytes under gradient and non-gradient conditions were studied with the Boyden micropore filter method using casein as a stimulant The in-filter distribution for 3 different incubation times, and one-level counts for a wide range of incubation times were determined photographically Under gradient (»chemotactic«) conditions, the number of responsing granulocytes depended on the sharpness of the casein gradient, whereas the average migrational velocity of the population appeared to be constant. It was not possible, by comparison with a theoretical model, to demonstrate a directional migration towards increasing casein concentrations. The previously reported Gaussian distribution under non-gradient (chemokinetic) conditions, was confirmed In these experiments the number of responding cells appeared to depend on the absolute casein concentration, while their average velocity seemed uninfluenced

Key words Leukocytes, human granulocytes, locomotion, *in vitro*- chemotaxis, chemokinesis.

A Schreiner, Med Dept. B, N-5016 Haukeland sykehus, Norway

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Studies concerning the locomotor response of leukocytes to environmental stimuli may yield information relevant to the nature of their migrational properties Knowledge of the kinetics of whole cell populations is also important for the evaluation of results obtained with any method for measuring chemotaxis

Zigmond & Hirsch (1973), using the Boyden millipore filter method (*Boyden* 1962) found that horse granulocytes – when moving in a non-gradient system – closely followed the behaviour expected from a random diffusion process for particles moving out from a thin layer as described by *Jacobs* (1967) These authors also found that the early responding portion of a granulocyte population, when moving up a gradient of a leukocyte derived factor, migrated *en masse*, showing a peak of cell concentration moving through the filter. This finding was considered evidential for the chemotactic property of the leukocyte factor. *Rosen* (1976) presented a mathematical model for chemotaxis based on results obtained by *Wilkinson* (1974) with the leading front method (*Zigmond & Hirsch* 1973, *Wilkinson* 1974). In this model the migration of granulocytes in filters is described by a moving normal distribution. To test these theories on whole cell populations, in-filter counts in various systems were made after a series of incubation times, and the results compared to theoretical models.

MATERIAL AND METHODS

Human leucocytes were isolated by dextran sedimentation in blood from laboratory staff members and medical students. The experiments were performed with modified Boyden chambers and 3 µm pore size millipore filters as described previously (*Schreiner*, in press), with the modification that leukocytes were delivered to the filters by sedimentation The upper compartments of the chambers were always filled with 0 85 ml of a cell suspension with 0 25 × 10^7 leukocytes in Hanks' balanced salt solution (HBSS) containing 2 per cent human serum albumin (HSA)

Gradient (chemotaxis) experiments. Cells in HBSS and HSA were placed above the filter, and casein Hammarsten 4, 2 or 1 mg per ml in HBSS was placed beneath the filter.

Non-gradient (activated random migration – chemokinesis) experiments. In addition to cells in HBSS and HSA above the filters, casein in the same concentration – 4,2 or 1 mg per ml – was placed both above and beneath the filter.

Non-gradient (not activated random migration) experiments. Cells in HBSS with HSA were placed above the filter and only HBSS beneath the filter.

Experimental Design

For chemotaxis and chemokinesis, 3 experiments were done for each casein concentration (4, 2 and 1 mg per ml): In each experiment 36 chambers were incubated simultaneously. *Every 20 minutes, 3 chambers were* removed and the filters immediately fixed. For random migration (not activated), 3 experiments were done· In each of these experiments 18 chambers were incubated, and the first 3 chambers were removed after 20 minutes and then 3 chambers every 40 minutes.

One-level counts. In each filter from each experiment, cells that had migrated into the filter were photographed and counted in 4 random fields at one level, 30 µm from the cell starting side.

In-filter distribution. The cell distribution within filters *after 20,60 and 180 minutes incubation* was determined in 3 chemotaxis and 3 chemokinesis experiments using 2 mg per ml casein, and in 3 random migration experiments with HBSS alone: In each of the triplicate filters, 2 random fields were photographed and counted on 5 different levels beginning 15 µm from the cell starting side, and advancing in steps of 15 µm.

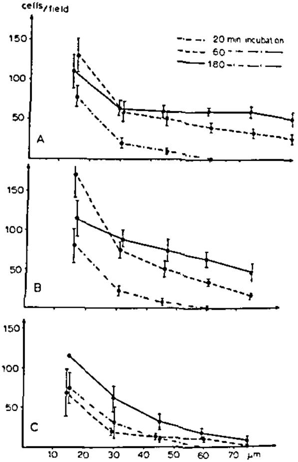


Fig. 1 A, B and C. Granulocyte counts at 30 µm from the cell-starting side in Boyden filters incubated for different periods of time in A: gradient, B activated, non-gradient, and C: not activated, non-gradient experiments. Mean of 4 random fields in each of triplicate filters from 3 experiments.

RESULTS

The cell distribution within filters after 20,60 and180 minutes incubation for gradient, activated non-gradient and not activated non-gradient experiments is shown in Figs. 1a, b and c. Figs 2a, b and c show the one-level counts from each type of experiments plotted against the corresponding incubation time. Because the resulting curves for separate individuals had equal shapes and showed maximal counts which in each case increased with increasing casein concentration, means were also calculated across the individual means. This procedure increased the clarity of the illustration at the expense of an increased standard error (SE). The standard deviation (SD) for individual means rarely exceeded 20% of the mean.

In experiments where cells had reached the distal surface, the proportion of neutrophils was consistently above 95%.

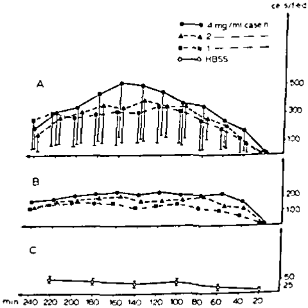


Fig 2 A, B and C In-filter distribution of granulocytes after 3 different incubation times in A: gradient, B activated, non-gradient, and *C* not activated, non-gradient experiments Mean of 2 random fields in each of triplicate filters from 3 experiments

Theoretical Approach

When particles move at random in a homogenous substrate from an initial distribution represented by a thin layer in position $x = 0$ at time $t = 0$, the distribution at a later time will be Gaussian, as indicated by *Jacobs* (1967):

$$n(x,t) = A(t)\, N \exp(-x^2/4\, \mu t) \qquad (1)$$

This equation for the expected number of particles per unit volume is based on a simplified model with constant mobility μ for all particles at all values of x and t, and a constant number N of particles per cross-sectional area. $A(t)$ is a normalization factor which is either $A(t) = A_2(t) = (4\pi\ \mu t)^{-1/2}$ for a medium which is infinite in both directions, or $A(t) = A_1(t) = (\pi\ \mu t)^{-1/2}$ for a semi-infinite medium

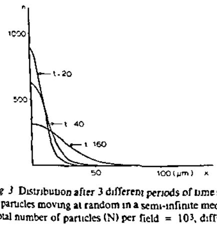


Fig 3 Distribution after 3 different periods of time (*min*) of particles moving at random in a semi-infinite medium Total number of particles (N) per field = 10^3, diffusion constant (μ) = 2 $\mu m^2 min^{-1}$

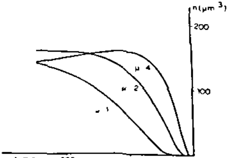


Fig 4 Time-dependence of particle concentration at 30 µm from the starting point for random diffusion with different mobilities. Diffusion constant (μ) = 1, 2 and 4

with a boundary at $x = 0$ In this model for simple diffusion, the highest concentration of particles will at all times be found near the thin layer (Fig. 3). If the concentration of particles is observed for a cross section in position x = constant = 30µm, the time-dependence for different mobilities will be as shown in Fig. 4. The t-axis has been reversed to give the impression a movement from the left to the right. When a directional force is added to this simple model by assuming a constant velocity v, the solution to the transport equation in an infinite medium is (*Rosen* 1967):

$$n(x,t) = A_2(t)\, N \exp -(x-vt)^2/4\mu t \qquad (2)$$

This function describes a propagating normal distribution.

Zigmond & Hirsch (1973) demonstrated that the distribution of granulocytes in millipore filters closely followed the model for random diffusion (equation (1), Fig. 3) *Rosen* (1976) succeeded in fitting results obtained in millipore filters by *Wilkinson* (1974) to the model represented by equation (2). Rosen's model implies that the medium is infinite in both directions. A more realistic model for filter experiments requires the exclusion of negative x-values, because the cells can only move into the filter. A model with this property can not be defined by a simple, exact equation, but a good approximation can be obtained by modifying the normalization factor in equation (2) to ensure a constant number of particles from $x = 0$ to $x = \infty$:

$$A(t) = A_3(t) = 1 \Big/ \int_{x=0}^{x=\infty} \exp\left[-(x-vt)^2/4\mu t\right]$$

For $v = 0$, $A_3(t)$ equals $A_1(t)$ and equation (2) becomes identical to equation (1).

Based on this model, the concentration profile at different times for a realistic choice of values for N, μ and v, is shown in Fig 5. The time-dependence for particle numbers in a cross-section at $x = 30\ \mu$ is shown in Fig. 6 for 3 realistic combinations of μ and v. Again, the time axis has been reversed. For $v = 0$, *i.e.* no directional force, the same curve as in Fig 4. ($\mu = 2\mu m^2\ min^{-1}$) is obtained.

Provided our simple model is adequate, the observed time-concentration profile (Fig. 2b and c) should be comparable to the curves shown in Fig. 4.

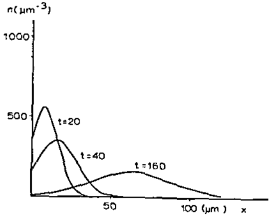


Fig. 5. Distribution of moving particles after 3 different periods of time (min) when the mobility results from random diffusion and an additional, directed force. Total number of particles (N) per field = 10^3, diffusion constant (μ) = 2 μm²min⁻¹, velocity resulting from directed force (v) = 0.2 μm min⁻¹.

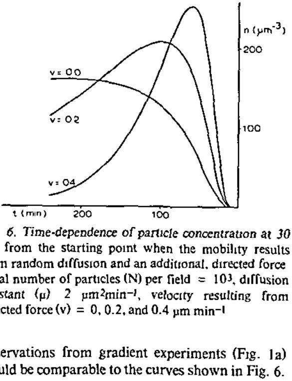


Fig 6. Time-dependence of particle concentration at 30 μm from the starting point when the mobility results from random diffusion and an additional, directed force Total number of particles (N) per field = 10^3, diffusion constant (μ) 2 μm²min⁻¹, velocity resulting from directed force (v) = 0, 0.2, and 0.4 μm min⁻¹

Observations from gradient experiments (Fig. 1a) should be comparable to the curves shown in Fig. 6.

DISCUSSION

The vast majority of *in vitro* measurements of leukocyte migration is done in millipore filters with various modifications of Boyden's method (*Boyden* 1962). Based mainly upon the observations made by *Zigmond & Hirsch* (1973), it is commonly accepted that random migration as well as directed migration can be observed and measured in millipore filter systems. It is also widely accepted that the migration observed in filter systems with a chemoattractant gradient is the resultant of random migration on one hand, and an additional, directed migration on the other (*Zigmond & Hirsch* 1973; *Wilkinson* 1974).

The Gaussian distribution of cells throughout the filters in non-gradient experiments was confirmed in our experiments. However, the *en masse* migration reported in gradient experiments by *Zigmond & Hirsch* (1973) could not be reproduced by us. A peak of cell concentration moving through the filter was never observed, although numerous experiments of the kind shown in Fig. 1a were done with different casein concentrations over a large range of incubation times (not shown). The presence of such a phenomenon as described in the experiments of *Zigmond & Hirsch* would have left the filter almost void of cells if the incubation time had been increased from 30 to 90 minutes.

The combination of data from filters which have been incubated for different periods of time (Fig. 2a, b and c) as an attempt to simulate the ideal situation of following the numbers of cells as a function of both time and space, is open to question: Differences in cell load from filter to filter will cause variations in the in-filter counts and influence the shape of the resulting curves. Furthermore, for longer incubation times »surface border problems« may arise: When a random type of migration is considered, the cells will move in all possible directions. They may reach the distal surface, but may not be able to find their way back into the filter. This effect of a limited medium would lead to a dilution of the cell concentration within each filter and reduce the in-filter cells count. If it holds true that random migration is a part of the chemotactic response, gradient experiments would also be influenced

Variations due to differences in cell load do occur (*Schreiner*, in press). However, despite the disturbance likely to arise from these variations, a well recognizable curve shape was reproduced from experiment to experiment. A dilution in the cell concentration near the distal surface of the filters may reduce the infilter count, but it is unlikely that this would alter the shape of the curves We therefore find it acceptable to compare the combined data with the theoretical functions shown.

Data from activated and not activated random migration (Fig. 1b and c) which were expected to follow the behaviour of a random diffusion, and thereby to be comparable with the transformed Jacob's formula (equation (2), Fig. 4), did so for the empirically realistic diffusion constant of 2 μm²sec⁻¹. Data from gradient experiments (Fig 1a) which – if the theory of a moving normal distribution holds true – should compare with the modified Rosen's function (equation (4), Fig. 6) did not do so, but compared favourably with the

theoretical model for random migration (equation (1), Fig 4) with a diffusion constant of 4 $\mu m^2 sec^{-1}$.

The finding that the one-level counts in all filters from chemotaxis and chemokinesis experiments (Figs 2a and b) increased with increasing casein concentrations, indicates that the number of responding cells is dependent on the sharpness of the gradient in gradient experiments, and on the absolute concentration in activated non-gradient experiments. In gradient experiments the cell counts were maximal at approximately 150 minutes for each casein concentration. The curves for different casein concentrations showed no displacement along the time axis, indicating that the average velocity of the moving granulocyte populations was fairly equal in each case. This result is in contrast to the finding of *Wilkinson* (1974), that increasing the sharpness of the gradient across the filter makes granulocytes move faster. An explanation may be that *Wilkinson* used the leading front method With this method the measured variable is the distance moved by the »fastest« 2–3 cells. In the case of a random type of movement, this distance will be a function of the number of responding cells, as well as of the distribution of mobilities in the cell population.

The failure to demonstrate whole population kinetics which apply to Rosen's model on one hand, and the failure to observe any displacement along the time axes for different gradients on the other, raise serious doubts as to whether a directional migration of granulocytes can be demonstrated in millipore filter systems.

From the »checkerboard analysis« of Boyden experiments made by *Wilkinson* (*Wilkinson* 1974, p 36) might be suspected that the contribution of gradients to the distance migrated by the leading front is modest However, because the contribution to the migrated distance of a directed force is proportional to time, and the contribution of a random movement proportional to the square root of time, such judgements are difficult The mathematical treatment of the checkerboard data by *Rosen* (1976) is more informative Based on his analysis it can easily be calculated that the contribution of the sharpest gradient to the migrated distance was approximately 30%, and less for weaker gradients and shorter incubation times. In summary the following conclusions can be drawn from our observations:

In millipore filter experiments with a gradient of casein, the number of responding granulocytes increased with the increasing sharpness of the casein gradient, while the average velocity of the different granulocyte populations within the gradient group was not influenced.

Although the granulocytes moved faster in gradient than in non-gradient systems it was not possible by comparison with a theoretical model to demonstrate directed migration of granulocytes towards casein in the millipore filter experiments. The locomotor behaviour of the granulocytes in filters under casein-gradient conditions, could be better described as an enchanced random migration. In casein-activated non-gradient experiments, the number of responding granulocytes depended on the absolute casein concentration, whereas their average velocity did not

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The skilled technical assistance by *Elin Askeland* and *Britt Edvardsen* is thankfully acknowledged

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Acta path microbiol scand. Sect. C, *86*: 211–214, 1978.

PROTEIN A REACTIVITY OF WHOLE RABBIT IgG AND OF FRAGMENTS OF RABBIT IgG

CURT ENDRESEN

The Gade Institute, Department of Microbiology, University of Bergen, Bergen, Norway

Endresen, C Protein A reactivity of whole rabbit IgG and of fragments of rabbit IgG. Acta path microbiol scand Sect C, 86 211–214, 1978

Approximately 15 per cent of the total IgG in normal rabbit serum reacted with protein A. When this protein reactive IgG fraction was treated with acid at pH 2 7 and then digested with plasmin, thus splitting the rabbit IgG between the C_H2 and C_H3 domains, both the isolated Facb and the pFc' fragments were protein A non-reactive This results indicates that at least one intact Fc chain is necessary to fulfil the protein A–Fc interaction Traces of protein A reactive rabbit $F(ab')_2$ fragments isolated from normal IgG were also found These fragments mixed with normal rabbit IgG precipitated protein A

Key words Rabbit IgG, staphylococcal protein A

C Endresen, Mikrobiologisk avdeling, MFH-bygget, N-5016 Haukeland sykehus, Norway

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IgG of most mammalian species interacts with staphylococcal protein A through the Fc region (7, 8, 9, 10, 11, 12, 13). *Kronvall et al.* (14) suggested that the precipitation reaction between protein A and human IgG was in two steps: A primary reaction between protein A and the Fc portion of the immunoglobulin, then a secondary protein – protein interaction They showed that the secondary reaction could be altered by carbamylation without any influence on the primary reaction Thus, by this chemical modification, human immunoglobulin lost the precipitation ability but the modified proteins were still able to inhibit the precipitation reaction between protein A and precipitating IgG (14).

In the »star-phenomenon« (15), the $F(ab')_2$ fragments involved were found to be fixed to a Sepharose-protein A column (6), indicating that these $F(ab')_2$ had specificity for protein A. The results showed that in human sera, there exist traces of IgG able to combine with protein A through their $F(ab')_2$ structure. This reactivity is apparently of bivale[illegible] being formed with monc

In contrast to human IgG, normal rabbit IgG does not precipitate with protein A but forms soluble complexes. This work examines the possible difference between human and rabbit IgG with respect to protein A reactivity.

MATERIALS AND METHODS

Rabbit IgG was isolated from pooled sera by ammonium sulphate precipitation, ion-exchange chromatography on DEAE-cellulose columns and gelfiltrated through Sephadex G-200 columns as described in (16) Rabbit IgG with protein A reactivity was isolated on a Sepharose-protein A column prepared and used as described in (5).

Plasmin was supplied as a lyophilized powder (3 casein units/mg protein Kabi AB, Sweden)

Plasmin digestion of IgG was performed as described in (1, 16) The pH of IgG (10–20 mg/ml) in saline was adjusted to 2.7 with 0.5 M HCl and the solution was incubated at 37° C for 15 min. The plasmin was added and the pH was readjusted to that optimal to the enzyme (1, 16) Digestion was performed with an enzyme-substrate ratio of 1:100 for 2 h at 37° C. The digestion mixture was either stored frozen (–20° C) or fractionated

immediately on a BioGel P-150 (BIO-RAD Lab., USA) column.

The BioGel P-150 column (2.5 × 88 cm) was equilibrated with 0.1 M sodium acetate buffer pH 4.6 containing 6 M urea. Fractionation was achieved with a flow-rate of 10 ml/h.

Immunoelectrophoresis and double diffusion analysis in agar were performed as before (2).

Antisera against rabbit IgG, Fc and pFc' were raised in guinea pigs. The animals were given 2 mg protein in 0.1 ml PBS mixed with 0.1 ml complete Freund's adjuvant (Difco, USA) into one foot-pad. After 14 days a similar dose was given intramuscularly, this time in incomplete Freund's adjuvant. After a period of 2 to 3 weeks, the animals were given 2–4 mg protein in 0.5 ml saline subcutaneously. After 14 days, the animals were bled by cardiac puncture. Also commercial goat anti-rabbit IgG serum (Behringwerke, BRD) were used.

Sodium-dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE) was performed in 12.5 per cent polyacrylamide as previously described (3).

RESULTS

Starting with 280 mg normal purified monomeric rabbit IgG, 34 mg were isolated on the Sepharose-protein A column. Thus, less than 15 per cent of normal rabbit IgG seem to be protein A reactive. Concentrated to 7 mg/ml in PBS, the protein A reactive fraction gave a precipitation in agar against protein A not observed using normal rabbit serum (Fig. 1). The isolated protein A reactive IgG was

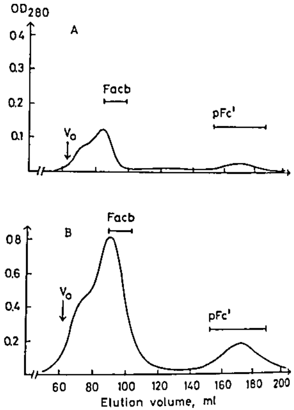


Fig. 2. A. Gel filtration on BioGel P-150 of a 2 h plasmin digest of protein A reactive normal rabbit IgG (12 mg/ml). Column size: 2.5 × 88 cm; V_o = 62 ml Elution buffer. 6 M urea – 0.1 M sodium acetate buffer pH 4.5. Elution rate: 9.4 ml/h. Facb and pFc' fragments was pooled as indicated in figure. B. Gel filtration of a 2 h plasmin digest of protein A non-reactive normal rabbit IgG (18 mg/ml). Other data as described above.

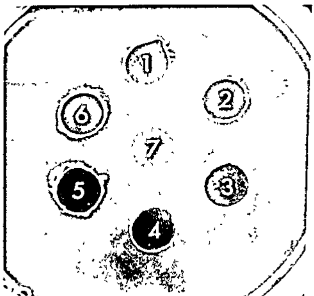


Fig. 1. Double diffusion analysis in agar shows the reactions between protein A (1 mg/ml) (well 7) and normal rabbit IgG protein A reactive fraction (7 mg/ml) (well 2), normal human serum (well 3) and normal rabbit serum (wells 1 and 4) Wells 5 and 6 contain PBS.

digested with plasmin, and the digest was then gelfiltered on a BioGel P-150 column (Fig. 2) under dissociating conditions (16). The fractions containing the Facb fragments (1, 16) were pooled as indicated in Fig. 2, and re-chromatographed on the same column. After extensive dialysis against PBS, the fragments were then applied to the Sepharose-protein A column. Most of the material passed the column in PBS, but some material was retarded and eluted by 3 M NASCN (about 2.8 mg). Analysis of this material, both non-reduced and reduced by SDS-PAGE, indicated the presence of Facb fragments together with intact IgG (Fig. 3).

After concentration, the protein A reactive fraction (Facb plus IgG) precipitated with protein A in double diffusion in agar. Pepsin digestion at pH 4 0, destroying all the Fc structure of the molecules, abolished the precipitation activity of the material However, traces of $F(ab')_2$ fragments were retained on the Sepharose-protein A column, indicating the presence of some specific antibody activity against protein A in normal rabbit IgG After dialysis and lyophilization, these $F(ab')_2$ fragments were dissol-

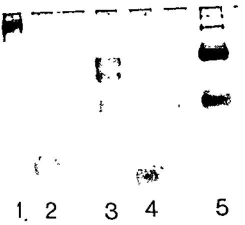


Fig 3 SDS-PAGE analysis of rabbit Facb fragment (1), pFc' (2), reduced and alkylated protein A reactive fraction (Facb plus intact IgG) (3), reduced and alkylated pFc' (4) Reduced and alkylated rabbit IgG was run as a marker (5)

ved in a few drops of normal rabbit serum This mixture produced a weak diffuse precipitation in double diffusion in agar against protein A.

Gelfiltration on Sephadex G-200 in 5 M guanidine HCl-1 M HAc, of totally reduced and alkylated samples of the protein A non-reactive fraction showed 2 peaks, one containing the light chains (Mol wt. 22,500) and the other reduced heavy chains free of the C_H3 region (Mol wt 42,000) The protein A non-reactive fraction thus consisted of almost pure Facb fragments (Fig 4)

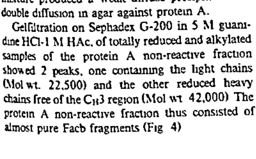


Fig 4 Gel filtration on Sephadex G-200 of reduced and alkylated rabbit IgG (——) and reduced and alkylated protein A non-reactive Facb fragments (- - - -) Column size 1 5 × 45 8 cm. V_o = 24 9 ml Elution rate 1 7 ml/h Eluant 5 M guanidine HCl – 1 M acetic acid. Double diffusion analysis shows the reaction between goat anti-rabbit IgG serum and intact rabbit heavy chain (3), the heavy chain of the Facb fragment lacking the C_H3 region (2), and pFc' (1)

DISCUSSION

Less than 15 per cent of normal rabbit IgG was protein A reactive by using a Sepharose-protein A column. In contrast to normal rabbit serum, the protein A reactive normal rabbit IgG in a concentration of 7 mg/ml, was able to precipitate with protein A in double diffusion. However, the reaction was weak compared to the reaction between normal human serum and protein A (Fig. 1). The protein A reactive IgG was eluted from the Sepharose-protein A column with 3 M NaSCN solution. Some denaturation of the rabbit IgG was observed. To a certain degree this may explain the weak and rather diffuse precipitation. However, similar treatment of normal human IgG still gave a stonger precipitation line with protein A than did rabbit IgG, when the same concentration of both human and rabbit IgG was used.

It is obvious that most of the protein A reactivity of normal rabbit IgG as well as that of human IgG is located at the Fc part of the IgG molecule (5, 8, 9, 10). As earlier described (2, 3), strong indications are found that at least in human IgG, an intact Fc chain is necessary to fulfil the requirements of the protein A–Fc interaction.

Since nearly all Facb fragments being produced by plasmin digestion of the protein A reactive rabbit IgG are protein A non-reactive, the requirement for one intact Fc chain for protein A interaction seems to be true also in the case of rabbit IgG The isolated rabbit pFc' fragments were all protein A non-reactive.

Traces of Facb fragments together with undigested IgG molecules were fixed to the Sepharose-protein A column. Pepsin digestion of this fraction, followed by a new immunoadsorption on the same column, showed that some $F(ab')_2$ fragments were protein A reactive.

In a previous paper *Grov & Endresen* (6) showed that traces of $F(ab')_2$ fragments isolated from normal human IgG and from normal guinea pig IgG did react with protein A and also that these fragments could replace specific rabbit antistaphylococcal $F(ab')_2$ fragments in the »star« formation. No activity was found in $F(ab')_2$ isolated from normal rabbit IgG. The discrepancy between this and previous observations most probably is due to the different amount of normal rabbit IgG used.

Obviously, the amount of specific $F(ab')_2$ activity of normal rabbit IgG against protein A is lower than that found in normal IgG. This fact, together with the observation that only 12–15 per cent of the normal rabbit IgG react with protein A (compared to about 90 per cent of human and guinea pig IgG), can explain why normal rabbit serum does not precipitate protein A in double diffusion in agar in

contrast to normal human and guinea pig serum.

In human serum, sub-classes IgG_1, IgG_2 and IgG_4 react with protein A, whereas IgG_3 is reported to be protein A non-reactive (12). Different sub-classes of rabbit IgG are not known, but papain-sensitive and papain-resistant forms of rabbit IgG have been shown (4). When protein A reactive rabbit IgG was digested with papain (*Endresen*, unpublished data) and plasmin, and this was compared with digested pooled IgG, no difference was observed. However, the protein A reaction can be of importance in the attempt to identify sub-classes of normal rabbit IgG.

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PROTECTION AGAINST EXPERIMENTAL *PROTEUS MIRABILIS* PYELONEPHRITIS IN RATS AND SIGNIFICANCE OF IMMUNITY

PETER LARSSON, BERTIL KAIJSER, SANTE OLLING and ANNICA SOHL ÅKERLUND

Departments of Immunology and Clinical Bacteriology, Institute of Medical Microbiology and Institute of Pathology, University of Goteborg, Goteborg, Sweden

Larsson, P., Kaijser, B., Olling, S & Sohl Åkerlund, A: Protection against experimental *Proteus mirabilis* pyelonephritis in rats and significance of immunity Acta path microbiol scand Sect. C, 86· 215–220, 1978

Intraperitoneal immunization with formalin-killed bacteria as well as previous hematogenous infection with *Proteus* O3H1 showed a protective effect against hematogenous pyelonephritis in rats when the homologous strain was used Transfer of hyperimmune antisera protected against hematogenously induced infection Neither intravesical or intraperitoneal immunization with formalin-killed bacteria nor transfer of urines containing antibodies of the IgG class protected against ascending pyelonephritis when the O3H1 strain was used Data are presented indicating that a rise of pH might decrease the biological effect of antibodies, suggesting that *Proteus* urease activity is a virulence factor of importance in this context

Key words P mirabilis, pyelonephritis, rats, immunity; protection

P Larsson, Institute of Medical Microbiology, Guldhedsgatan 10, S-413 46 Goteborg, Sweden

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Urinary tract infection (UTI) is a common problem in children Most of these infections are caused by *Escherichia coli*. The second most common causative agent is *Proteus*. Such bacteria are often found in the presence of complicating factors such as malformations of the urinary tract, neurogenic bladder disorders or a history of several previous infections (5, 19)

Since recurrent UTI often occur in spite of antibiotic treatment, the use of prophylactic immunization against the infecting bacteria has been contemplated (10) Several investigations have used animal models to study the role of immunity in the urinary tract (for reviews see e g 12,23). For *E. coli* these investigations have demonstrated that serum antibodies can protect against both retrograde and hematogenously induced pyelonephritis (3, 4, 11, 14, 16, 17, 20, 29, 32) Most recently our studies have indicated that also urine antibodies may prevent ascending pyelonephritis in rats (16)

As a continuation of our studies concerning protection against infection caused by *E. coli*, the present investigation was carried out to compare and analyse the possibilities of achieving protection against *Proteus* infection in the urinary tracts.

MATERIAL AND METHODS

Bacterial Strains

Proteus mirabilis and *vulgaris* strains belonging to the serological system of *Kauffmann & Perch* (18) and with the following O and H antigens were used *(Kauffmann & Perch designation in parenthesis)*: O1H1 (XL), O3H1 (XK), O3H2 (F 403), O6H1 (F 181), O7H1 (F 27), O10H3 (F 73), O10H4 (P 506), O28H3 (F 87) All strains except O1H1 (XL) were *P. mirabilis* strains

Animals

Female Sprague-Dawley rats weighing 200–300 g (Anticimex, Stockholm, Sweden) were used. They were given pellets and tap water ad libitum

contrast to normal human and guinea pig serum.

In human serum, sub-classes IgG_1, IgG_2 and IgG_4 react with protein A, whereas IgG_3 is reported to be protein A non-reactive (12). Different sub-classes of rabbit IgG are not known, but papain-sensitive and papain-resistant forms of rabbit IgG have been shown (4). When protein A reactive rabbit IgG was digested with papain (*Endresen*, unpublished data) and plasmin, and this was compared with digested pooled IgG, no difference was observed. However, the protein A reaction can be of importance in the attempt to identify sub-classes of normal rabbit IgG.

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PETER LARSSON, BERTIL KAIJSER, SANTE OLLING and ANNICA SOHL ÅKERLUND

Departments of Immunology and Clinical Bacteriology, Institute of Medical Microbiology and Institute of Pathology, University of Goteborg, Goteborg, Sweden

Larsson, P, Kaijser, B, Olling, S & Sohl Åkerlund, A Protection against experimental *Proteus mirabilis* pyelonephritis in rats and significance of immunity. Acta path microbiol scand Sect C, 86: 215–220, 1978

Intraperitoneal immunization with formalin-killed bacteria as well as previous hematogenous infection with *Proteus* O3H1 showed a protective effect against hematogenous pyelonephritis in rats when the homologous strain was used. Transfer of hyperimmune antisera protected against hematogenously induced infection. Neither intravesical or intraperitoneal immunization with formalin-killed bacteria nor transfer of urines containing antibodies of the IgG class protected against ascending pyelonephritis when the O3H1 strain was used Data are presented indicating that a rise of pH might decrease the biological effect of antibodies, suggesting that *Proteus* urease activity is a virulence factor of importance in this context.

Key words *P mirabilis*, pyelonephritis, rats, immunity, protection.

P Larsson, Institute of Medical Microbiology, Guldhedsgatan 10, S-413 46 Goteborg, Sweden

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Urinary tract infection (UTI) is a common problem in children Most of these infections are caused by *Escherichia coli*. The second most common causative agent is *Proteus* Such bacteria are often found in the presence of complicating factors such as malformations of the urinary tract, neurogenic bladder disorders or a history of several previous infections (5, 19)

Since recurrent UTI often occur in spite of antibiotic treatment, the use of prophylactic immunization against the infecting bacteria has been contemplated (10) Several investigations have used animal models to study the role of immunity in the urinary tract (for reviews see e g 12,23) For *E coli* these investigations have demonstrated that serum antibodies can protect against both retrograde and hematogenously induced pyelonephritis (3, 4, 11, 14, 16, 17, 20, 29, 32) Most recently our studies have indicated that also urine antibodies may prevent ascending pyelonephritis in rats (16)

As a continuation of our studies concerning protection against infection caused by *E. coli*, the present investigation was carried out to compare and analyse the possibilities of achieving protection against *Proteus* infection in the urinary tracts.

MATERIAL AND METHODS

Bacterial Strains

Proteus mirabilis and *vulgaris* strains belonging to the serological system of *Kauffmann & Perch* (18) and with the following O and H antigens were used (*Kauffmann & Perch* designation in parenthesis): O1H1 (XL), O3H1 (XK), O3H2 (F 403), O6H1 (F 181), O7H1 (F 27), O10H3 (F 73), O10H4 (P 506), O28H3 (F 87) All strains except O1H1 (XL) were *P. mirabilis* strains.

Animals

Female Sprague-Dawley rats weighing 200–300 g (Anticimex, Stockholm, Sweden) were used They were given pellets and tap water ad libitum.

TABLE 1. *Antibodies to* Proteus *O3 and H1 Antigens as Measured with ELISA in (1) Sera from Healthy Rats, (2) Hyperimmune Sera Used for Passive Transfer of Antibodies in Hematogenous* Proteus *O3H1 Pyelonephritis and (3) Sera from the Rats Actively Immunized with Formalin-killed O3H1 Bacteria before Hematogenous O3H1 Pyelonephritis. Mean Values and Ranges are Given*

Sera obtained from group	No of rats	O3 antibodies			H1 antibodies		
		IgG	IgA	IgM	IgG	IgA	IgM
1	4	7 (5–12)	24 (19–27)	7 (5–9)	32 (26–38)	22 (18–31)	25 (20–31)
2	6	65 (48–80)	105 (44–150)	78 (30–114)	96 (57–119)	83 (41–110)	74 (16–101)
3	7	48 (35–63)	54 (38–92)	79 (67–92)	72 (59–89)	46 (32–61)	28 (21–41)

Immunization Procedures

I. *Active immunization* Formalin-killed (0.5%) bacteria from broth cultures of the strains O1H1, O3H1, O3H2, O6H1 or O10H3 were given three times intraperitoneally in a dose of 0.25 ml (10^9bact/ml) at seven-day intervals. Intravesical immunization was carried out with the same preparations and time schedule by introduction of the antigen into the bladder via the urethra using a blunt needle. For intravesical immunization the strains O10H4 and O28H3 were also used.

Immunization was also carried out by causing hematogenous pyelonephritis (see below) with the O3H1 strain (29). After two weeks of infection the animals were treated with intraperitoneal injections of 20 mg ampicillin (Astra, Sodertalje, Sweden) given twice daily for one week to eradicate the infection Hematogenous challenge using the O3H1 strain was performed 60 hours after the last antibiotic dose. No ampicillin could at that time be detected in blood or urine samples of rats, as determined with the paper disc diffusion method (10μl of sample/disc, *Sarcina lutea* ATCC 9341, PDM-ASM-agar (Biodisk, Stockholm, Sweden))

II. *Passive intravenous immunization.* Sera were obtained from six rats immunized twice with formalin-killed O3H1 bacteria with an interval of one week and infected one week later with live bacteria of the same strain. These sera contained high levels of O3 and H1 antibodies (Table 1) as determined with the enzyme-linked immunosorbent assay, ELISA (6, 21). These sera were pooled and injected (0.5 ml) into the tail vein or intracardially into healthy rats two hours before hematogenous challenge with live O3H1 bacteria (see below) Control animals received 0.5 ml of normal pooled rat sera before infection.

III *Passive intravesical immunization* Pooled urine (0.25 ml) was injected into the bladder via the urethra just before intravesical infection with the O3H1 strain The following types of pooled urine samples were used

A urine from 15 healthy rats

B urine from five rats with acute pyelonephritis caused by the *O3H1 strain* and with urinary antibodies of the IgG class to *Proteus* O3 and H1 antigens

C urine from ten rats immunized intravesically with the O3H1 strain and with urinary antibodies of the IgG class to *Proteus* O3 and H1 antigens

For groups B and C urines were obtained from days 33–36 after initiation of the infection or the immunization The antibody levels of the urine pools were determined with the ELISA and are shown in Table 2.

TABLE 2 *Antibodies to* Proteus *O3 and H1 Antigens as Determined with ELISA in Pooled Urines from (A) Fifteen Healthy Preimmune Rats, (B) Five Rats with O3H1-induced Acute Ascending Pyelonephritis and (C) Ten Rats Immunized Three Times Intravesically with Formalin-killed O3H1 Bacteria*

Urine pool	O3 antibodies			H1 antibodies		
	IgG	IgA	IgM	IgG	IgA	IgM
A	6	33	38	3	12	7
B	9	26	14	10	12	7
C	15	37	16	8	13	7

Infection Procedures

I *Hematogenous infection* Live *Proteus* O3H1 bacteria (0 4 ml of 10^9bact/ml) were injected intracardially followed by kidney massage as described by *Sanford et al* (29)

II *Ascending infection* Live *Proteus* bacteria (0.5 ml of 10^9bact/ml) were injected into the bladder via the urethra using a blunt needle In the case of passive intravesical immunization, 0 25 ml of the urine pools A, B or C was injected just before infection with 0.25 ml of live bacteria (2×10^9bact/ml).

TABLE 3 *Protection against Hematogenous* Proteus *O3H1 Pyelonephritis in Rats after Intraperitoneal Immunization with Formalinized Bacteria or Intracardiac Injection of Live Bacteria*

Proteus strain used for Immunization		Total no of rats	Rats with no or minor pyelonephritis[a]
O3H1	(killed)	10	8[b]
O3H1	(live)	9	8[c]
O3H2	(killed)	10	1
O6H1	(killed)	10	3
non immunized		9	1[bc]

[a] healthy rats and rats with pyelonephritis of grade 1
[b] [c] $8 \neq 1$ ($p<0.01$)

Sampling

Serum and urine samples were obtained as described earlier (16)

Confirmation of Pyelonephritis

After killing the rats the kidneys were removed under sterile conditions and examined Cut surfaces were inoculated on to CLED agar plates (Oxoid) for non-swarming growth of *Proteus*. O grouping was performed on positive cultures as a control of infection (22) Microscopical examination was carried out as described earlier (16) Grading of renal abscesses from 0 to 4 was performed according to the method of *MacLaren* (24) 0 = no signs of pyelonephritis, 1 = one or more discrete pinpoint abscesses, 2 = several pinpoint abscesses showing coalescence, 3 = confluent lesions occupying less than half of the kidney, 4 = confluent lesions occupying more than half of the kidney

Antibody Determination and Isolation of Antigens

Determination of antibodies to *Proteus* O3 and H1 antigens was performed using the enzyme-linked immunosorbent assay, ELISA (6, 21) with rabbit anti-rat IgG, IgA and IgM from Nordic Laboratories, Tilburg, The Netherlands The O3 antigen was prepared from a boiled O3H2 preparation and used in an optimum concentration The H1 antigen was prepared from the O7H1 strain and used in a concentration of 0 1 g/l (21) The rabbit anti-rat sera were diluted 1/250 before use The antibody titres were defined as earlier described (16) and were expressed in per cent of standard sera Owing to the small amounts of urines available, the samples were diluted 1.2 when used for antibody determination

Influence of pH on Absorption of Antibodies with Formalin-killed Bacteria

Sera obtained on days 7 or 30 from five rats with O3H1-induced acute pyelonephritis were used for absorption experiments Formalin-killed O3H1 bacteria were added to the antisera diluted 1/10 in veronal buffer (0 05 mol/l, pH 7.2, 8 2 and 9 2) and incubated for 30 min at 37° C. After centrifugation, the O3 antibody levels were determined in the supernatants using the ELISA. The extinction values for antibodies of IgG, IgA and IgM immunoglobulin classes obtained for an absorbed serum sample at the different pHs were expressed in per cent of the value for the same untreated serum at the corresponding pHs

Statistics

Statistical comparison of the groups in Table 3 were performed using the Fischer test for testing equality of binomial proportions

Statistical comparison of the groups shown in Table 4 was performed by using a scores test (one-sided) designed for testing equality of two multinomial distributions against an ordered alternative

The influence of pH on antibody activity, Table 5, was statistically tested by combining the results from five independent Spearman rank correlation tests (one-sided) each corresponding to one animal

TABLE 4 *Protective Effect of Passively Transferred Serum Antibodies on Hematogenous Challenge with* Proteus *O3H1*

Rat serum given	No of rats	Result of bacterial challenge		
		death	pyelonephritis	healthy
Normal serum[a]	20	12	8	0
Hyperimmune serum[a]	20	2	15	3

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Immunization was also carried out by causing hematogenous pyelonephritis (see below) with the O3H1 strain (29) After two weeks of infection the animals were treated with intraperitoneal injections of 20 mg ampicillin (Astra, Sodertalje, Sweden) given twice daily for one week to eradicate the infection Hematogenous challenge using the O3H1 strain was performed 60 hours after the last antibiotic dose. No ampicillin could at that time be detected in blood or urine samples of rats, as determined with the paper disc diffusion method (10µl of sample/disc. *Sarcina lutea* ATCC 9341, PDM-ASM-agar (Biodisk, Stockholm, Sweden))

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III. *Passive intravesical immunization.* Pooled urine (0.25 ml) was injected into the bladder via the urethra just before intravesical infection with the O3H1 strain The following types of pooled urine samples were used

- A urine from 15 healthy rats
- B urine from five rats with acute pyelonephritis caused by the O3H1 strain and with urinary antibodies of the IgG class to *Proteus* O3 and H1 antigens
- C urine from ten rats immunized intravesically with the O3H1 strain and with urinary antibodies of the IgG class to *Proteus* O3 and H1 antigens

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Urine pool	O3 antibodies			H1 antibodies		
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A	6	33	38	3	12	7
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C	15	37	16	8	13	7

Infection Procedures

I *Hematogenous infection.* Live *Proteus* O3H1 bacteria (0.4 ml of 10^9bact/ml) were injected intracardially followed by kidney massage as described by *Sanford et al* (29).

II. *Ascending infection* Live *Proteus* bacteria (0 5 ml of 10^9bact/ml) were injected into the bladder via the urethra using a blunt needle In the case of passive intravesical immunization, 0 25 ml of the urine pools A, B or C was injected just before infection with 0.25 ml of live bacteria (2×10^9bact/ml)

At least part of this latter protective effect of urine could be eliminated by absorption with *E. coli* K antigen.

Investigations concerning the role of immunity in *Proteus* infections have shown that serum antibodies to *Proteus* can protect against hematogenously or intrarenally induced infections (28, 29). Protective effects of immunization have also been reported against ascending *Proteus* pyelonephritis. *McLaughlin & Domingue* have suggested that antibodies to the common enterobacterial antigen might be of importance (26), but this has not been verified. Previous hematogenous infection seems to prevent ascending *Proteus* UTI in rats when the same strain is used for challenge (11). In that study intraperitoneal immunization of boiled *Proteus* bacteria as well as intravenous transfer of hyperimmune sera seemed to have a protective effect.

In our studies active local immunization or passive transfer of urines with antibodies to *E. coli* O6K13H1 bacteria protected against ascending O6K13H1 pyelonephritis (16). Similar experiments with *Proteus* O3H1 showed no protective effect. One explanation may be the fact that *Proteus* have no recognizable K antigen which is regarded as an important virulence factor for *E. coli* (13, 15, 31). These antibodies to *E. coli* K antigens are efficiently protective in experimental infections (14,16, 17). Furthermore, the urines used in the *E. coli* experiments contained IgG and IgA antibodies (16), in contrast to the *Proteus* experiments, where only antibodies of the IgG immunoglobulin class could be demonstrated

In this study active immunization, but not passive transfer of antibodies, protected against hematogenous *Proteus* O3H1 pyelonephritis. This might be explained by an additive effect of antibodies to the O3, the H1 and possibly also to other antigens The lack of protective effect of antibodies to only one antigen might be due to differences in the amounts and possibly avidities of antibodies available for protection Other defence mechanisms such as cell-mediated immunity may also be of importance

Immobilizing antibodies, considered to be directed against *Proteus* flagellar antigens, have been attributed a role in resistance against UTI in rats (28) In the present as well as in other studies by us, no protective effect of H antibodies has been shown in experimental UTI caused by *Proteus* and *E. coli* (16, 17) In the *Proteus* experiments, however, only urine antibodies of the IgG class were detected. A possible role of pili in experimental *Proteus* pyelonephritis has been suggested, but so far no studies on protective immunization using pili have been reported (30).

The present study obviously illustrates differences between *E. coli* and *Proteus* infections regarding the host-parasite relationship. Apart from the antigens mentioned above, other mechanisms such as the urease production of *Proteus* can explain these dissimilarities. This enzyme, absent in *E. coli*, splits urea into ammonia and carbon dioxide, causing a *rise of the urinary pH*. The urine becomes supersaturated with magnesium ammonium phosphate and calcium phosphate. As a result, renal stones are formed (9) The rise of pH leads to necrosis of the renal tubular epithelium and also favours an intracellular localization of the infection to the tubular epithelium (2). Urinary leucocytes are considered to be lysed, probably due to the high urinary pH, in experimental *Proteus* UTI in rabbits (8).

Studies of experimental pyelonephritis caused by *Proteus* in mice and rats have demonstrated that urease-inhibitors reduce the ability of the bacteria to cause renal damage (1, 25, 27). Furthermore, alkalinity may influence the biological effect of antibodies present in the urine of the infected animals The experiments carried out in the present investigation support this theory since absorption of antibodies was less complete at higher pH. A dissociation of antigen-antibody complexes at alkaline pH has earlier been described for benzenearsonic acid hapten and its corresponding antibody (7)

In conclusion *Proteus* seems to have other virulence mechanisms than *E. coli* which are relevant for the capacity to cause UTI. Such information may be of significance when prophylactic immunization against UTI is considered.

The excellent technical assistance of *Lena Beijer, Gunvor Jefferth, Kerstin Larsson, Ingrid Sutherland* and *Marie Svensson* is gratefully acknowledged

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TABLE 5. *Serum Antibodies against* Proteus *O3 Antigen Remaining after Absorption with the same Number of Formalin-killed O3H1 Bacteria at Different pH. The Antibody Values Determined with ELISA were Expressed in per Cent of the Values Obtained for the Corresponding Untreated Serum*

Rat no	IgG antibodies			IgA antibodies			IgM antibodies		
pH	7.2	8.2	9.2	7.2	8.2	9.2	7.2	8.2	9 2
1	73	68	78	45	85	69	35	56	96
2	33	42	63	43	54	78	35	30	64
3	40	45	72	67	77	93	27	31	55
4	80	94	115	57	44	93	21	20	47
5	39	52	65	49	84	63	5	17	4
Mean	53	60	79	52	69	79	25	31	53

RESULTS

Protective Effect of Active Immunization on Hematogenous Pyelonephritis Caused by Proteus *O3H1*

After intraperitoneal immunization with formalin-killed *Proteus* O3H1 a protective effect was shown against hematogenous challenge with the homologous strain ($p<0.01$) (Table 3). Previous experimental pyelonephritis caused by O3H1 bacteria also showed a protective effect when the rats were challenged with the same strain ($p<0.01$). No protection against O3H1 bacteria could be demonstrated after immunization with formalin-killed O3H2 or O6H1 bacteria.

Protective Effect of Passive Immunization with Serum Antibodies on Hematogenous Infection Caused by Proteus *O3H1*

Intravenous or intracardiac injection of hyperimmune rat sera containing antibodies to *Proteus* O3H1 as shown in Table 1, protected against hematogenously induced infection ($p<0.05$) (Table 4) even if many rats acquired pyelonephritis. These hyperimmune sera contained about the same levels of antibodies to the O3 and H1 antigens as sera from the rats intraperitoneally immunized with O3H1 bacteria (Table 1) which protected from the hematogenous pyelonephritis described above.

Protective Effect of Active Immunization on Ascending Infection Caused by Proteus *O3H1*

Intraperitoneal or intravesical immunization with O1H1, O3H1 or O10H3 bacteria did not protect against ascending infection with the homologous strain. Neither did intraperitoneal or intravesical immunization with the heterologous strains O1H1, O3H2 or O10H3 protect against challenge with the O3H1 strain. Intravesical immunization with O10H4 or O28H3 strains had no protective effect on ascending O10H3 pyleonephritis. Ten or more animals were used in each group – altogether about 200 rats.

Protective Effect of Passive Immunization with Urinary Antibodies Against Ascending Pyelonephritis Caused by Proteus *O3H1*

Antibodies to *Proteus O3* and *H1* antigens of the IgG immunoglobulin class could be demonstrated in urine obtained from rats with acute pyelonephritis (Table 2, pool B) or intravesically immunized rats (pool C). Intravesical injection of twenty rats with either of the urine pools B or C, however, gave no significant protection against ascending pyelonephritis.

Influence of pH on Absorption of Antibodies with Formalin-killed Proteus *O3H1 Bacteria*

Absorption of antibodies to *Proteus* O3 antigen in rat sera with formalin-killed O3H1 bacteria was significantly more efficient at lower pH value when pHs 7.2, 8 2 and 9.2 were compared, as shown in Table 5 ($p<0.05$). This effect was significant for O3 antibodies of the IgG and IgA, but not significant for antibodies of the IgM immunoglobulin class.

DISCUSSION

Prophylactic immunization against experimental urinary tract infections (UTI) caused by *E. coli* in animals has shown that O and K antibodies in serum can protect against hematogenous pyelonephritis in rabbits and rats, as well as against ascending pyelonephritis in rats (3, 4, 11, 14, 16, 17, 20, 29, 32). Local immunization in the urinary tracts with formalin-killed *E coli* bacteria or intravesical administration of urine samples containing antibodies against the infecting *E. coli* strain can also mediate protection in the ascending rat model (16).

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URAEMIC TOXINS OF HIGH MOLECULAR WEIGHT INHIBITING HUMAN MONONUCLEAR PHAGOCYTES CULTURED *IN VITRO*

S JÖRSTAD and S. KVERNES

Jorstad, S & Kvernes, S. Uraemic toxins of high molecular weight inhibiting human mononuclear phagocytes cultured *in vitro* Acta path microbiol scand Sect. C, 86: 221–226, 1978.

Uraemic plasma and normal serum were separated into fractions of different molecular sizes by means of molecular filtration through Pellicon filters and liquid chromatography on Sephadex G-15. The amount of substance was considerably higher in uraemic fractions than in comparable normal fractions. When normal human mononuclear phagocytes were cultured in the presence of the uraemic fraction containing the largest molecules, detachment of phagocytes from the glass coverslips was significantly increased The digestion capacity of the remaining cells was decreased The results indicate that substances in uraemic plasma responsible for the impaired function of human mononuclear phagocytes cultured *in vitro* consist of molecules of a molecular weight higher than 10 000

Key words Uraemia, molecular weight; toxicity, mononuclear phagocytes, *in vitro*.

Storker Jorstad, University of Trondheim, Department of Medicine, Sections for Nephrology and Haematology, N-7000 Trondheim, Norway

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Since the publication of the middle molecule hypothesis in 1972 (1), clinical investigations of toxic serum substances in uraemia have been concentrated on the molecular weight (mwt) range from 500 to 5000 *In vitro* studies showed that fractions in the middle molecule range inhibited glucose utilization in rat diaphragm, brain and kidney cortex slices, as well as in human erythrocytes (5), caused a diminished granulocyte iodination capacity (8) and influenced the haemoglobin synthesis (6) An inhibitory effect of lymphocyte transformation has also been reported (2, 9) In the search for toxic molecules, it is important to have a standardized test model Using human mononuclear phagocytes, we were able to show that plasma from uraemic patients exerted significant inhibitory effects on the function of macrophages cultured *in vitro* (7) The purpose of the present work was to investigate the effect on the macrophages caused by substances in different molecular ranges obtained from uraemic plasma.

MATERIALS AND METHODS

Blood Samples

Uraemic plasma was obtained from non-fasting patients with serum creatinine higher than 900 µmol/l Some patients were on dietary treatment, others were treated with haemodialysis and from these only predialysis plasma was used. Heparin (Nyegaard & Co. A/S, Norway) was added to the samples in a concentration of 0 05 mg per ml The blood cells were spun down and the plasma stored in deep frozen state, if not used immediately. Pooled A-serum from healthy adults also with 0 05 mg heparin per ml added, was used as control

Separation of Uraemic Plasma and Normal Sera

Molecular filtration Uraemic plasma and normal serum were filtered through a 47 mm stirred cell system (Millipore, USA) connected with a pressure tank containing 99 9 per cent pure nitrogen With an operating pressure of 3 0 kg/cm^2, approximately 10 ml of plasma/serum was filtered in 4 hours. Pellicon filters (Millipore, USA) with different nominal molecular weight limits (nmwl) were used PSAC (nmwl 1000) PTGC (nmwl 10 000) and PSED (nmwl 25 000)

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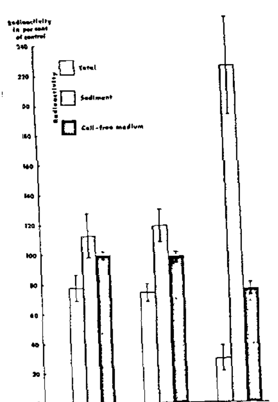


Fig 1 Effect on phagocytosis of radiolabelled *Candida albicans* by human mononuclear phagocytes cultured from the 4th to the 8th day in different permeates obtained by filtration of uraemic plasma through Pellicon filters (PSAC, PTGC, PSED) Total radioactivity reflects cell detachment during the culture period from the 4th to the 8th day, sediment the undigested Candida particles in cells detached during the test period from the 8th to the 9th day, and medium the digestion capacity of the phagocytes still attached on the 8th day of culture The values are the means ± S E M of the results from 18 experiments with plasma from 11 patients, expressed as percentages of controls (corresponding permeates obtained from normal serum) Each experiment was performed in triplicate culture dishes The range of the controls was 29-83 × 10^2 ct/min radioactivity per culture dish

Fig 2 shows a typical chromatogram of normal PSED (nmwl 25 000) filtered serum, and Fig 3 a corresponding chromatogram of PSED filtered uraemic plasma The reference substances ribonuclease -A, NAD⊕ and creatinine were eluted by the volumes of 58 ml, 88 ml and 140 ml, respectively

Table 1 shows the different peak areas determined as cm^2 per 10 ml filtered uraemic plasma and

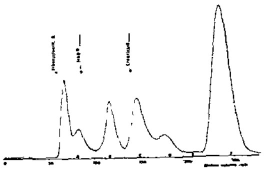


Fig 2 A typical chromatogram of normal serum with three reference substances, Ribonuclease -A mwt 13 500, NAD + mwt 663 5 and creatinine mwt 113 For specification, see text

normal serum, and the distribution of the substances in different molecular ranges within uraemic plasma and normal serum. It should be noted that even though peak A itself represents a larger area in uraemic plasma than in normal serum, it accounts for only 10 9 ± 2 0 per cent of the absorbing substances in uraemic plasma and for 15.8 ± 2.9 per cent in normal serum

Fig 4 shows the results when different fractions obtained from PSED (nmwl 25 000) filtered uraemic plasma on Sephadex G-15 were used as culture media. The results are expressed as percentage of controls, which are the corresponding fraction filtered from normal serum. Molecules in fraction A decreased the total radioactivity to 46.2 ± 7.6 ($p < 0.01$) and the radioactivity in the sediment to

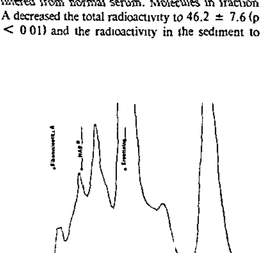


Fig 3 A typical chromatogram of uraemic serum with three reference substances, Ribonucleose -A mwt 13 500, NAD + mwt 663.5 and creatinine mwt 113. For specification, see text

Gentamicin 40 µg per ml was added to the permeate. Permeates with 25 per cent normal serum were used as culture medium. Plasma from 11 different patients were used in 18 experiments.

Gelfiltration

Normal serum and uraemic plasma filtered through Pellicon filters with nmwl 25 000 were subjected to gelfiltration. The chromatography was carried out on a 45 × 2.5 cm column (Jobling 108003/130) packed up to 36.0 cm with Sephadex G-15 (Pharmacia, Sweden). After equilibration of the column with 0.01 M Tris-HCl buffer, pH 7.15, 10 ml of filtered uraemic plasma of normal serum was applied to the top of the column. The column was eluted with 0.01 M Tris-HCl buffer ph 7.15, using a peristaltic pump (LKB, 12 000 Varioperpex) and a outflow rate of approximately 1.4 ml/min. The absorption was measured at 254 nm, using Biorad 1300 u.v. detector and an Omni Scribe$_{TM}$ recorder (Houston Instruments, USA) operating at a constant speed of 10 cm per hour. The fractions (A-F) were collected manually at eluation volumes as shown in Fig. 2 and Fig. 3. Each fraction was evaporated in vacuum at 37°C to a volume of about 5 ml. In order to get the same concentration of the substances as in the start volume of 10 ml, the fractions were diluted according to the formula: evaporated fraction (ml) + sterilized water (ml) + 2.5 ml normal serum = 10 ml. The molality was adjusted to 280 mosm/l with 10 per cent NaCl and pH to 7.4 with 1 M NaOH or 1 M HCl. Each fraction from uraemic plasma was used as culture medium with the corresponding fraction from normal serum as control, both with 25 per cent normal serum. Plasma from seven different patients were used in 14 experiments Three reference substances were used: Ribonuclease -A, mwt 13 500 (Sigma No R4875), NAD⊕, mwt 663.5, (Sigma No 7004) and creatinine, mwt 113 (Fluka AG, Buchs SG No 168033-24) The peak areas were determined planimetrically on the chromatograms and expressed as cm^2 per 10 ml plasma/serum

Cell Culture

The methods for culturing human mononuclear monocytes and for testing the phagocytosis of heat-killed radiolabelled *Candida albicans* have been reported in detail previously (10, 11) Monocytes were separated from venous blood obtained from healthy donors, as described by *Boyum* (4) After washing, the mononuclear cells were suspended in the medium RPMI 1640 (Gibco, Scotland) supplemented with 25 per cent pooled human A-serum (complete medium) in concentration of 4 × 10^6 cells per ml. The solution was dispensed in volumes of 0 3 ml on glass coverslips (Leighton type 10 × 22 mm) in petri dishes (Costar 3506, USA) (two coverslips in each petri dish). After an incubation period of 90 min, the medium containing non-adherent cells (lymphocytes) was removed and 1.0 ml of fresh complete medium was added to each culture dish. The medium was changed on the first and the fourth day of culture Prepared fractions were used as culture media from the 4th to the 8th day of culture. At the end of the incubation period on the 8th day, the media were removed and each petri dish was incubated for 15 min with 1.0 ml complete medium containing 2 × 10^6 radiolabelled Candida cells per ml The coverslips were then washed six times in complete medium and placed in new petri dishes, after which fresh complete medium was added. The cultures were harvested after 24 hours. The medium was centrifuged at 2000G for 10 minutes and the radioactivity on each coverslip, in the medium and in the sediment was registered, using a Wallac GM gamma radiation counter (Finland)

Total radioactivity means the sum of radioactivities registered on the coverslips, in the sediment and in the cell-free medium in each culture dish Compared to the controls, the total radioactivity reflects cell detachment when the cells were under influence of uraemic plasma fractions from the 4th to the 8th day of culture The radioactivity found in the sediment reflects the amount of undigested Candida particles in macrophages detached during the test period from the 8th to the 9th day of culture. After phagocytosis, the macrophages extrude the debri of digested Candida particles to the cell-free medium, and the radioactivity found here reflects the digestion capacity of the macrophages still attached on the 8th day.

Statistics

Each experiment was performed in triplicate culture dishes. The results are given as the mean ± standard error of the mean (S.E.M.). P-values were calculated using the Wilcoxon test for paired differences

RESULTS

Fig. 1 shows the effect on human mononuclea macrophages cultured in permeates from uraemi plasma filtered through Pellicon filters with diffe rent nmwl. The results are expressed as percentag of controls which are the corresponding permeate achieved from normal sera. With molecules up to nmwl of 1000, the total radioactivity was decreasec to 77.9 ± 8.9 ($p < 0.01$), the radioactivity in the sediment increased to 112.4 ± 14 3 (n.s.) and the radioactivity in the cell-free medium decreased to 99 5 ± 2.8 per cent (n s.) With molecules up to nmwl of 10 000, the total radioactivity was reduced to 74.6 ± 5 8 ($p < 0.01$), the radioactivity in the sediment increased to 118.3 ± 10.4 (n.s.) and the radioactivity in the cell-free medium decreased to 97.3 ± 3 2 per cent (n.s).

Using molecules up to nmwl of 25 000 in the culture medium, the total radioactivity was reduced to 29.7 ± 8 2 ($p < 0.01$) The radioactivity in the sediment increased to 222.6 ± 32.3 ($p < 0.01$) and the radioactivity in the cell-free medium decreased to 75.2 ± 4.0 per cent of the controls ($p < 0.01$) The range of the controls was 29-83 × 10^3 ct/min radioactivity per culture dish.

used as culture medium from the 4th to the 8th day of culture, a significant detachment from coverslips was registered during the culture period. The digestion capacity and detachment of the remaining cells, however, was not significantly influenced. Permeates after filtration through PSED filters (nmwl 25 000) used as culture medium depressed the macrophage function, including both detachment and digestion capacity, almost to the same extent as we found when using unfractionated uraemic plasma (7).

It should be emphasized that Pellicon filters do not have sharp molecular range cut-off. The retention by filtration depends on molecular size and not on mwt. The results obtained with Pellicon filters indicate, however, that molecules in uraemic serum responsible for the impaired phagocyte function have molecular weights which are higher than 10 000. Even though the detachment from the 4th to the 8th day was significantly increased when using PSAC (nmwl 1000) and PTGC (nmwl 25 000) permeates, the detachment was much more pronounced with PSED (nmwl 25 000) permeates. As the digestion capacity only was reduced by serum fractions with molecular weights higher than 10 000, it might be that one substance is responsible for cell detachment and another larger substance is responsible for depressing digestion capacity.

The fact that NAD$^{\oplus}$ appeared with an elution volume of 88 ml on the Sephadex G-15 filtration reflects that peak A may contain molecules from the middle molecular range up to the cut-off of the PSED (nmwl 25 000) filter.

Only peak A caused an inhibitory effect on the macrophage function, roughly to the same extent as found when using PSED (nmwl 25 000) permeates as culture medium. Substances in peaks containing smaller molecules (B-F) exerted no depressive effect. No exact data can be given about the molecular sizes of the substances in peak A, but when the results from the Pellicon filtration are compared with those of the Sephadex G-15 filtration, it seems reasonable that the toxic substance or substances in peak A have a *mwt which is higher than the »middle molecular«* range (500–5000).

Accumulation of substances does not necessarily mean that they exert a toxic effect. The amount of substances is larger in every uraemic peak than in the normal ones. As the UV-absorbtion may vary from one substance to another, the different peak areas give no quantitative information about the concentration of unknown substances within the same chromatogram. Our findings do not conflict with those of *Bergström et al.* (3) who found that a middle molecule fraction (peak 7c) exerted an inhibitory effect on *in vitro* lymphoblastic transformation. It might be that some substances have toxic effect on lymphoblastic transformation and others on human mononuclear phagocytes cultured *in vitro*.

The search for uraemic toxins is difficult because we are dealing with substances of unknown molecular size and of unknown concentration and intracorporeal distribution. Moreover, it is even more problematic whether substances of different molecular weights act synergistically in producing uraemic toxicity. Our results indicate, however, that the toxins which exert a depressive effect on human mononuclear phagocytes cultured *in vitro* have a larger mwt than the »middle molecule« range.

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TABLE 1. *Peak Areas (cm²/10 ml Normal Serum and Uraemic Plasma) of Fraction A-F, and each Fraction Expressed as Percentage of the Total Area Corresponding to the UV Absorbing Material Eluted from Sephadex G-15-Separated Uraemic Plasma (314.2 ± 17.6 cm²) and Normal Serum (94.1 ± 11.6 cm²)*

Fraction	A		B		C		D		E		F	
	Area cm^2	%	Area cm^2	%	Area cm^2	%	Area cm^2	%	Area cm^2	%	Area cm^2	%
Uraemic	33 2 ± 5 2	10 9 ± 2.0	54 0 ± 5 0	17.5 ± 1 6	130 3 ± 15.1	40 5 ± 3 2	28 2 ± 2 8	9 0 ± 1 0	6 8 ± 1 9	2 3 ± 0 4	61 8 ± 6 0	19 9 ± 1
Normal	16 7 ± 4 3	15 8 ± 2 9	9 1 ± 2 2	8 8 ± 1 4	11 7 ± 1.7	12.7 ± 1 9	6 5 ± 0 5	7 9 ± 1 2	1 0 ± 0 2	1 1 ± 0 2	49 0 ± 6 1	51 6 ± 3

The values given are the means ± S.E.M. of the results from separation of 14 uraemic plasma and 9 normal sera

162.8 ± 20.3 ($p < 0.01$). The radioactivity in the cell-free medium decreased to 74.0 ± 3.7 per cent ($p < 0.01$). The range of the controls was 25-59 × 10^2 ct/min radioactivity per culture dish. The other fractions (B-F) did not alter the macrophage function.

DISCUSSION

Plasma from uraemic patients has been shown to exert a depressive effect on the macrophage function (7). This investigation was carried out in order to see whether this effect could be related to a certain molecular range in uraemic plasma. Two different techniques for molecular separation were used molecular filtration with Pellicon filters and gel filtration on Sephadex G-15.

In order to give the cell cultures adequate growth conditions, all fractions (normal and uraemic) were supplemented with 25 per cent normal serum It has been shown previously that it is immaterial whether plasma or serum is used as culture medium (7). When uraemic plasma filtered through PSAC (nmwl 1000) and PTGC (nmwl 10 000) filters was

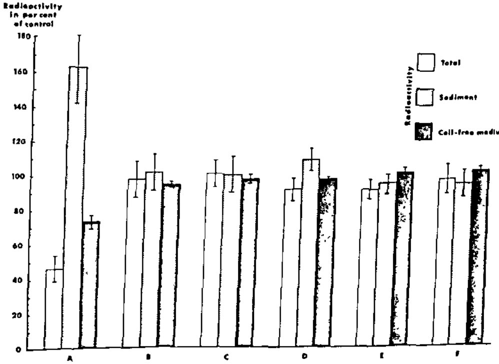


Fig. 4. Effect on phagocytosis of *radiolabelled Candida albicans by human mononuclear phagocytes* cultured from the 4th to the 8th day in different uraemic plasma fractions (peaks A-F) obtained by Sephadex G-15 filtration Total radioactivity reflects cell detachment during the culture period from the 4th to the 8th day, sediment undigested Candida particles *in* cells *detached* during the test period from the 8th to the 9th day, and medium the digestion capacity of the phagocytes still attached on the 8th day of culture. The values are the means ± S E.M. of the results from 14 experiments with PSED filtered plasma from seven patients, expressed as percentage of controls (*corresponding fractions* obtained from normal serum). Each experiment was performed in triplicate culture dishes The range of the controls was 25-59 × 10^2 ct/min radioactivity per culture dish.

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THE REACTIVITY OF HUMAN PLATELETS IN THE ^{125}I-LABELLED STAPHYLOCOCCAL PROTEIN A TEST

RIITTA KEKOMÄKI

Finnish Red Cross Blood Transfusion Service, Helsinki, Finland

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Stored platelets have been employed in the testing of various antiplatelet antibodies and platelet unrelated immune complexes The antisera included sera containing ABO-, HLA- or platelet specific (Zwa) antibodies and serum from an adult patient with idiopathic thrombocytopenic purpura (ITP) Use was made of ^{125}I-labelled staphylococcal protein A (IPA) for the detection of platelet bound IgG The reactivity of cells with the ITP serum and sera from healthy blood donors increased after storage for a few days, and declined after a highly reactive period extending over some weeks In some cases predilution of the serum increased the probability of discovering weak antibodies The normal components of the serum modified the platelet-antibody reaction, but all the various kinds of platelet reactive activities could be detected by this single test.

Key words Platelets, immune complexes, staphylococcal protein A, radioimmunoassay

Riitta Kekomaki, Finnish Red Cross Blood Transfusion Service, Kivihaantie 7, SF-00310 Helsinki 31, Finland

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The aim of this study is to characterize the interaction of stored human platelets and various antibodies by a single test. the platelet-^{125}I-labelled protein A (PIPA) test (12). A rabbit immune complex serum, characterized previously in the platelet aggregation sedimentation pattern test (13), and human gammaglobulin polymers were used as additional references.

MATERIALS AND METHODS

Separation and Preservation of Platelets

Human venous blood from healthy volunteers of blood group O (or A, if indicated) was collected in blood bags (Code No FPR 0512, Travenol Laboratories S A , Castlebar, Country Mayo, Ireland, citrate-phosphate-dextrose (CPD) anticoagulant)

From 40 to 60 buffy coats were harvested in an equal volume of acid citrate-dextrose-NaCl (ACD-NaCl, 1·5, v/v) anticoagulant. The platelets were then separated by differential centrifugation. The cells were washed four times in EDTA-Tris-saline (0 009 M *ethylene-diaminetetra-acetate* (EDTA), 0 067 M Tris-hydroxymethylamino-

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THE REACTIVITY OF HUMAN PLATELETS IN THE ^{125}I-LABELLED STAPHYLOCOCCAL PROTEIN A TEST

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Three ml of platelet suspension was treated with neuraminidase (from *Clostridium perfringens* N 9130, 30–50 U/mg, Sigma Chemical Corp., St. Louis, MO., USA) for 60 min at 37°C, pH 7.0. The minimum final activity of the enzyme was adjusted to 0.1 or 0.01 U/l × 10^9 platelets. The treatment with trypsin (8213, 130 000 U/g, Merck AG, Darmstadt, W. Germany) was carried out at 37°C for 30 min. The enzyme activity was adjusted to 200 or 40 U/l × 10^9 platelets. For retardation of the reactions and the removal of traces of enzymes, the platelets were washed twice with ten volumes of cold (+4°C) CBS.

RESULTS

A. *Normal and Pathologic Sera, Specific Reagents and Isolated Serum Fractions in the PIPA Test*

A.1. Effect of normal human sera. The amount of direct binding of iodinated protein A on to washed platelets varied only slightly, even when different batches of platelets were utilized.

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As a rule, duplicates gave results which fell within a narrow range. The test was repeated if the results showed a difference of more than 20 per cent. The reproducibility of the results obtained in respect of normal sera was high ($r = 0.64$, $n = 12$). In addition, repeated measurements with different platelet preparations and different lots of

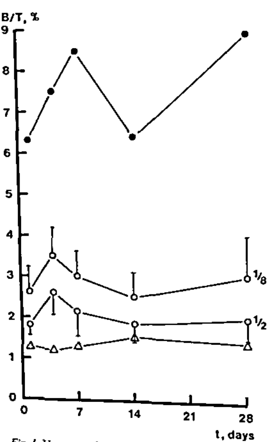


Fig. 1. Variation of the IPA-binding related to the age of one platelet preparation. The mean and 1 S.D. (n = 14) are indicated for the two dilutions of normal sera. Symbols: open circle = normal serum, solid circle = ITP serum and triangle = platelet control.

methane (Tris, Sigma Chemical Corp., St. Louis, MO., USA), 0.1 M saline, pH 7.0, and once with citrate buffered saline (CBS, 0.006 M citrate, 0.15 M saline, pH 7.2) before final suspension in the same buffer. Microscopic examination was made to ascertain the purity of platelet preparations. NaN_3 (Merck AG, Darmstadt, W. Germany) was added to a final concentration of 0.01 per cent.

A proportion of each fresh platelet preparation was fixed with 4 per cent formaldehyde, as described by *Allain et al.* (1). After three washings with physiological phosphate buffered saline (PBS; 0.093 M phosphate 0.15 M saline, pH 7.4), the preparation was treated with an equal volume of 1 M aqueous ethanolamine (Fluka AG, Buchs SG, Switzerland) for one hour in order to bind the remaining aldehyde groups (24). Finally, NaN_3 was added as above. The platelets were preserved in small capped aliquots at + 4°C. These were used at a mean age of approx. four weeks, if not otherwise stated. Small samples of some batches were frozen rapidly in a cold bath at −20°C without cryopreservative and used immediately after thawing at 37°C.

Radio-iodination of Protein A

Protein A (PA) from *Staphylococcus aureus* was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and carrier-free $Na^{125}I$ from the Radiochemical Centre Ltd., Amersham, England. The iodination of PA was effected by the chloramine-T method (9), as modified by *Dorval et al.* (6). An amount of 20 µl of the preparation contained approximately 4×10^5 cpm, and the specific activity of the iodinated PA (IPA) was approximately 10 µCi/µg IPA was stored at −20°C and used within six weeks.

Test System

The platelets were suspended in a solution of low ionic strength (LIS), (15), with a conductance of 3.7 mmho at 37°C, and an osmolality of approx 305 mosm/kg) The suspension contained 1×10^6 platelets/µl, as calculated in a Coulter Counter model Fn. The aperture diameter was 70 µm Varying threshold values were also employed for estimation of the relative number of platelet aggregates in test suspension

Various dilutions of the sera were tested, the most commonly used being 1 8. To 0.1 ml of serum dilution was added an equal volume of platelets, and incubation was carried out for one hour at an ambient temperature. After two washings with one ml of CBS, 20 µl of IPA was added. The incubation time was half-an-hour at ambient temperature. The platelets were then washed twice with CBS and resuspended in one ml of the same buffer. A sample of 0.9 ml was transferred to a counting vial and counted for 30 s

The test could also be made on microtitre plates with 96 U-bottom wells of 250 µl (Meda, Helsinki) A quantity of 15 µl of cell suspension was added to 20 µl of serial serum dilutions. The mixtures were incubated for 60 min at an ambient temperature. Two washings were made, with aliquots of 150 µl CBS per microwell, and 20 µl of IPA diluted 1:4 was added to each well. Incubation for 30 min at ambient temperature was followed by two washings and resuspension in 150 µl CBS. Finally, samples of 100 µl were transferred to counting vials The results are expressed as the percentage of the total ^{125}I added which was bound by the platelets (bound/total) All assays were carried out in duplicate.

Sera and Reagents Used to Characterize the PIPA test

In each test setting, the following three controls were included.

(1) platelets incubated with CBS instead of serum (platelet control);

(2) a normal serum, selected for representation of the mean value of 14 sera obtained from healthy blood donors (normal serum control);

(3) one control serum obtained from an adult patient with a clinical diagnosis of idiopathic thrombocytopenic purpura (ITP). This serum had been tested for the presence of immune complexes by the platelet aggregation sedimentation pattern test (19) with negative result The undiluted serum or serum diluted 1:2 reacted weakly in a microcytotoxicity test (2) with anti-HLA-B 15 related specificity.

The same serum displayed a weak but specific binding of IPA in dilution 1:8 which exceeded the random variance of the binding induced by normal sera (Fig 1) The ability of the test to distinguish between this positive serum and the normal serum was taken as the descriminative ability of the test. The titre of the serum is expressed as the highest dilution of the serum which exdeeds the IPA-binding induced by normal serum at the particular dilution in which its maximal binding is displayed.

The following other specific reagents were included as indicated:

(1) One normal rabbit serum,

(2) Rabbit serum, known to contain in vivo induced platelet-unrelated soluble immune complexes (rabbit-IC), was used for identification of the IC-binding sites (Fc-receptors) on platelets (13),

(3) For the same purpose, purified human gammaglobulin (16%; Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) was aggregated with heat (63°C for 10 min). Immediately after centrifugation (3 000 g/30 min), the soluble polymers (heat-aggregated IgG, HA-IgG) were separated with acrylamide agarose gel-filtration (Ultrogel AcA 22, LKB Ltd, Industrie Biologique Francaise) in Tris-HCl buffer (0 1 M Tris-HCl, 0 2 M NaCl, 0.05% NaN_3), pH 7.6. It was found that the isolated polymers reacted with platelets in the platelet aggregation sedimentation pattern test (19), even at a concentration of 1 µg/µl (unpublished data);

(4) Human immune anti-A serum obtained from the Finnish Red Cross Blood Transfusion Service. This had a specific antibody titre in excess of 2500, as determined by the indirect Coombs agglutination technique employing red blood cells,

(5) Platelet-specific antiserum (containing C-fixing anti-Zw^a antibodies) generously supplied by Professor *A Svejgaard*, Tissue Typing Laboratory, Rigshospitalet, Copenhagen, Denmark;

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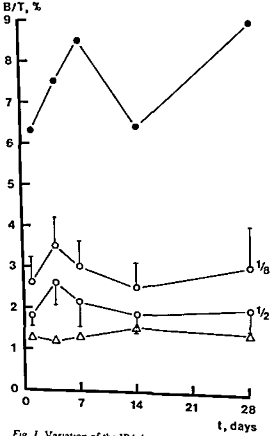


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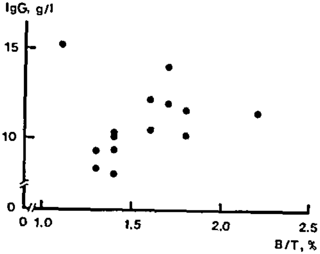


Fig. 2. Positive correlation between the IPA-binding induced by normal serum diluted 1:8 and the concentration of IgG in the undiluted serum (n = 13; r = 0.59).

IPA showed that none of the normal sera augmented IPA binding to the specific binding level evaluated in every test setting by the weakly positive serum (ITP).

The amount of IgG attached to the platelets and the potent of fixing IPA might be a function of the total amount of serum IgG. When tests were made with undiluted serum or even serum diluted to one half, no correlation was detectable between the IgG concentration of serum and the binding of IPA. When serum diluted 1.8 was examined, there was correlation (r = 0.59, n = 13) between IPA binding and IgG (Fig. 2).

A.2. Effect of pathologic sera and specific reagents. In some instances, the discriminative ability of the test was found to be enhanced when serial dilutions of serum were tested. In particular, when ITP-serum or anti-HLA serum was diluted 1 4 to 1.8, the binding of IPA by the platelets was promoted At higher dilutions, the binding diminished. More avid or concentrated antibodies, anti-Zw^a, and anti-A or HA-IgG, gave the most marked reaction when used undiluted (Fig. 3). The rabbit serum, rich in IC, reacted in a similar manner The IPA binding was seen to decline with increasing serum dilutions. When dilution was plotted against binding, the slope varied individually for different sera (Fig. 3).

A.3. Effect of isolated serum fractions Anion-exchange chromatography (QAE-Sephadex) was performed for concentration of the IgG fraction from the serum. Both IgG and IgG-dS were tested for reactivity. In ITP serum, the presumed antiplatelet antibody was generally found in the IgG fraction (Table 1). In accordance with this, the platelet-associated activity was seen to move with the 7 S marker in density-gradient centrifugation. Minimal antiplatelet activity was located in the 19 S region (Fig. 4), as measured by the PIPA test.

Different behaviour was observed with rabbit serum containing in vivo induced platelet unrelated immune complexes. As regards the fractions of ion-exchange chromatography, the main activity of unfractionated serum was in IgG-dS (Table 1). In density gradient centrifugation, it sedimented as fast as or faster than the 19 S marker, as reported earlier (12). To minimize the bias resulting from the possible formation of aggregated gammaglobulin during the chromatography, normal human or rabbit sera were carried as simultaneous controls for the ITP and rabbit IC-sera, respectively. No more than traces of platelet reactive activity were observable in the IgG fractions of normal human or rabbit sera (Table 1).

B. *Role of the Platelets in PIPA Test*

B.1. Age of the platelet preparation When the platelets were tested six hours after blood donation, they were unable to discriminate between the weak specific and non-specific reactions (Table 2). It was found that even one day's storage augmented the binding, and storage of from three to four days resulted in further enhancement of the binding of IPA by the platelets (Fig. 1).

During a period of one to three subsequent weeks (Fig. 1), the sensitivity of the PIPA test remained essentially unchanged. The increment in the binding of IPA coincided with a spontaneous formation of platelet aggregates Their number increased during the first 3–4 days of preservation. Subsequently, the ratio of single cells to aggregates remained almost stable, the aggregates representing approximately 30 per cent of the total number of particles. A small decrement in the number of aggregates was observable after a storage period of several weeks.

An increase was discernible in the non-specific binding from normal serum during succeeding months. Mainly for this reason, the discriminative ability of the test weakened (Fig. 5). The specific binding ability of cells was not affected by further storage. The platelets were found to be reactive even after storage for ten months.

B.2. ABO-group of platelets The amount of platelet-bound IgG from ITP-serum was independent of the blood group of the platelets (Table 3)

B.3. Reactivity of platelets fixed with formaldehyde Platelets treated with formaldehyde displayed properties essentially similar to those of platelets stored in sodium azide alone. However, the direct IPA-

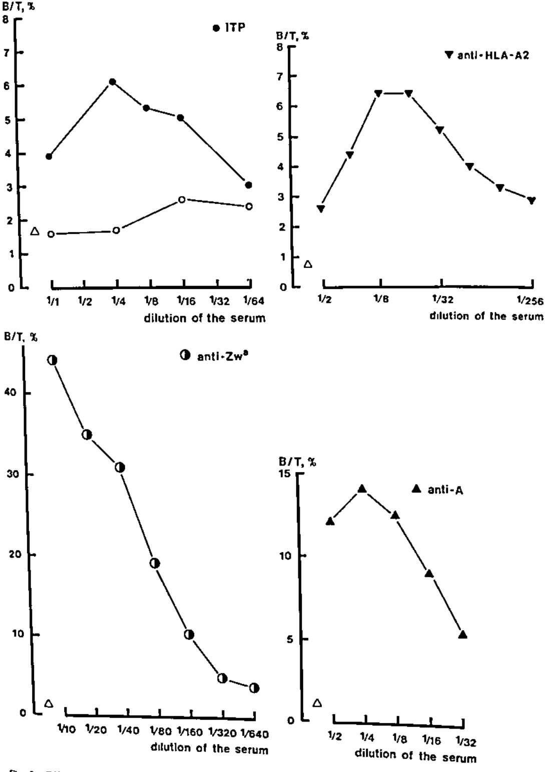


Fig 3a Effect of dilution of the serum on IPA-binding induced by various antisera Symbols as in Fig 1 and as indicated in separate figures

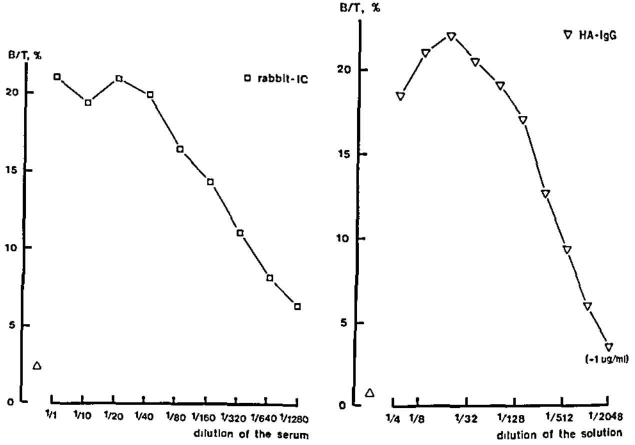


Fig. 3b. Effect of dilution of the serum on IPA-binding induced by rabbit immune complex serum (rabbit-IC) or heat-aggregated gammaglobulin (HA-IgG). Protein concentration of the undiluted HA-IgG = 3.84 g/l Symbols. triangle = platelet control. Other symbols as indicated in separate figures.

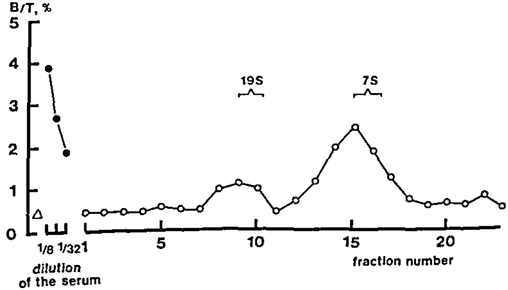


Fig. 4. Fractionation of the ITP serum by density gradient centrifugation. *The inset (solid circle) gives the IPA-binding of the serum dilutions indicated. The fractions (open circle) were tested as 1:2 dilutions.*

TABLE 1. *IPA-binding Activity of the IgG and IgG-depleted Fractions of Human and Rabbit Sera Fractionated by Ion-Exchange Chromatography*

Type of serum			cps	Protein concentration g/l
Normal human	diluted	1:8	241	
		IgG	296	2.1
		IgG-dS	118	6 5
ITP patient	diluted	1.8	440	
		IgG	814	2.6
		IgG-dS	496	15 8
Normal rabbit	diluted	1.8	205	
		IgG	196	1.0
		IgG-dS	87	5 1
Rabbit-IC	diluted	1.8	1170	
		IgG	186	1.0
		IgG-dS	1272	6 5
Platelets			96	

Total counts added per test tube approx 8300 cps
ITP, idiopathic thrombocytopenic purpura
Rabbit-IC, rabbit serum containing immune complexes formed in vivo.

binding of the platelets was increased, with a diminished discriminative ability as the net result (Table 3).

B 4 Reactivity of platelets stored in frozen state. The discriminative ability of the test for ITP serum remained almost unaltered if the platelets were stored either in sodium azide or in frozen state The Fc-receptors were preserved in both forms of storage The reactivity of the platelets was weakened if the cells were frozen and thawed three times.

B 5 Reactivity of platelets treated with enzymes The number of single cells in test suspension was diminished after treatment with any of the enzymes

TABLE 2 *Non-reactivity of a Fresh Platelet Preparation*

Type of serum	Dilution of serum	Age of platelet preparation < 1 day	16 days
		cps	cps
Normal human	undiluted	51	48
	diluted 1 8	73	176
ITP patient	undiluted	63	–
	diluted 1 8	70	481
Platelets		80	84

Total counts added per test tube approx 7900 cps
ITP, idiopathic thrombocytopenic purpura

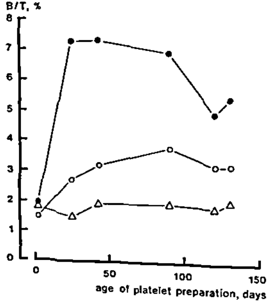


Fig 5 Variation of the IPA-binding related to the age of six different platelet preparations in one simultaneous testing. Symbols as in Fig 1.

In experiments with neuraminidase, the binding from normal or ITP serum, both undiluted and diluted, was enhanced (Table 4a). Trypsin treatment of the platelets diminished the dilution-induced enhancement of IPA-binding by normal and ITP-

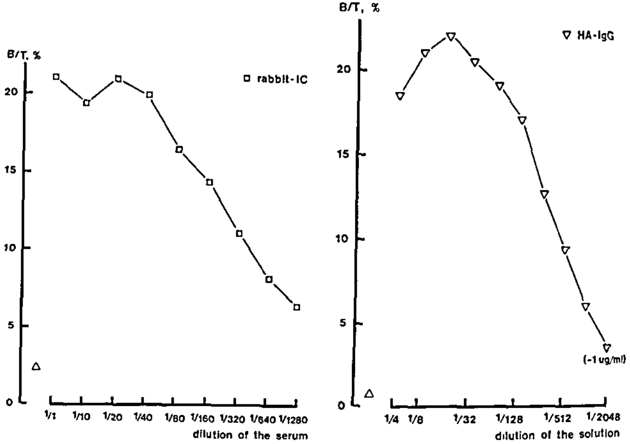


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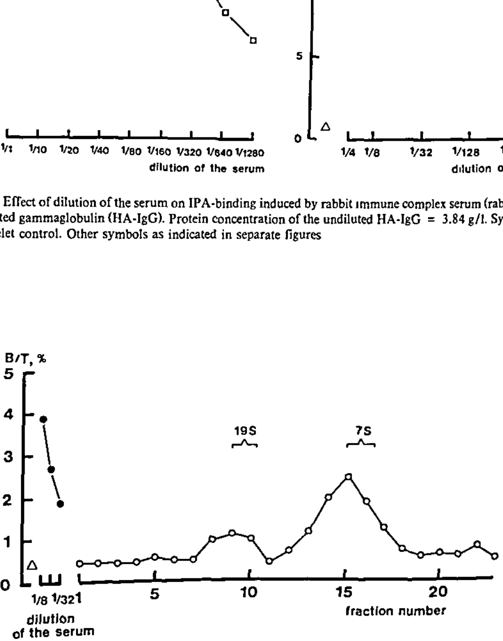


Fig 4. Fractionation of the ITP serum by density gradient centrifugation. The inset (solid circle) gives the IPA-binding of the serum dilutions indicated. The fractions (open circle) were tested as *1:2* dilutions.

mediated reactions between unrelated immune complexes and platelets have been used to characterize the measuring properties of platelets in this test system.

The reactivity of the platelets in the test, as characterized by the reaction of the weakly positive and normal sera, showed a constant pattern which was dependent on the age of cells. The initial hyporeactivity of the first few days, exploited by *Svejgaard* (23), was followed by a more reactive period It is known that platelets display irreversible shape changes even after 24 hours at + 4°C (4). It is possible that swelling and partial lysis make accessible antigenic sites and receptors that were previously unexposed In 1954, *Moreau & André* suggested that only a minor part of the A- or B-antigen is present on the surface of the platelet, and that the major part is more deeply located (16) In agreement with this concept, in the present series good binding of anti-A was demonstrable in platelets even after months of storage. The period of high reactivity lasted for several weeks. Finally, although the change to increased reactivity coincided with cessation of the spontaneous formation of platelet aggregates, the aggregation *per se* could not explain the alteration in reactivity without simultaneous membrane changes in single cells.

Attempts were made to induce changes in the reactivity of platelets by the treatment of fresh cells with enzymes Treatment with neuraminidase, with only one hundredth of the enzyme activity reported by *Glynn & Phil* (7), led to enhancement in the IPA binding ability of the test cells It is known that treatment with neuraminidase diminishes the serotonin binding capacity (7). It could accordingly uncover receptors which were previously masked To preclude false positive reactions, treatment with neuraminidase has not been used in routine testing to date

The enhancement in binding attained with trypsin-treated platelets is in agreement with the results obtained by *Pfueller & Lüscher* (21) They suggested that the proteolytic digestion of platelets unmasked Fc receptor sites on them In addition, the enzymatic digestion of complement receptors could eliminate the possibility of these binding sites for preference being occupied by normal serum components

The binding from undiluted normal serum, or serum diluted to one-half, was not found to correlate with the total amount of IgG in the serum. When the normal control serum was diluted to 1·8, the binding of IPA was enhanced. A definite correlation now existed between the IPA-binding and the concentration of IgG in serum. A similar enhancement of IPA-binding by serum dilution was discernible for weak specific reactions (12). This corroborates the assumption that, in addition to IgG, some other factor(s) control the binding of antibody on to platelets in undiluted serum.

All the specific control sera could be titrated in the PIPA test, as suggested previously by us (12), and the results compared quantitatively. To date, this has been impracticable when using a single technique. The search for optimal binding conditions of weak antiplatelet antibodies necessitates extended investigations.

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sera (Table 4b). None of the treatments exerted more than a minimal effect on the binding of IPA to platelets induced by rabbit IC-serum.

TABLE 3. *IPA-binding of Various Control Sera in Dilution 1:8 as Studied with Platelets Preserved in Two Different Ways and of Two Different Blood Groups*

Type of serum	Method of preservation			
	Azide		Formaldehyde treatment and azide	
	Blood group		Blood group	
	A	O	A	O
Normal human	198[a]	190	274	234
anti-A	1102	–	1085	–
ITP patient	407	553	404	380
Platelets	76	69	254	238

ITP, idiopathic thrombocytopenic purpura.
[a]) cps
Total counts added per test tube approx. 7500 cps.

DISCUSSION

Stored washed platelets are adaptable (3, 5,12, 17, 22) to the detection of platelet-associated IgG with a view to avoiding the monitoring of the metabolic changes in fresh cells (8, 11, 20). The use of stored platelets has circumvented some additional problems encountered with fresh preparations. The daily separation of platelets has been precluded, and as pooling has been made feasible, the variation induced by interindividual differences in cells could be minimized. Although the possible bias caused by HLA allotypia cannot be eliminated, the very rare antigens are presumably diluted by pooling.

As specific marker of platelet-associated IgG, ^{125}I-labelled staphylococcal protein A was taken. This is known to react with Fc-parts of all human IgG subgroups except IgG_3, and to react with rabbit IgG (6). In this study, the non-reactivity of IPA with IgG_3 is of no more than marginal significance, as the results obtained with a few known sera were compared throughout the study.

As it is known that human platelets carry several types of membrane antigens and immunological receptors (i.e., *ABO-*, *HLA-* and *platelet specific* antigens and Fc-receptors), both specific antibody reactions with platelets and what are termed Fc-

TABLE 4a *Effect of Treatment of Platelets (1 × 10⁹ Cells) with Neuraminidase (0 1 U or 0 01 U for 60 Minutes at 37°C, pH 7 0) on IPA-binding and on Number of Platelets in Test Suspension*

Type of serum	Dilution of the serum	Neuraminidase 0 1 U	0 01 U	control
Normal human	1 2	179[a])	118	96
	1 8	274	211	188
ITP patient	1 2	736	725	294
	1 8	910	1016	509
Rabbit-IC	1 20	1077	1373	1004
Patelets		165	136	130
Total number of platelets:		514	603	905
Number of aggregates:		246	295	409

[a]) cps
Total counts added per test tube approx. 7700 cps.
ITP, idiopathic thrombocytopenic purpura.
Rabbit-IC, rabbit serum containing immune complexes formed in vivo.

TABLE 4b. *Effect of Treatment of Platelets (1 × 10⁹ Cells) with Trypsin (200 U or 40 U for 30 Minutes at 37°C, pH 7.0) on IPA-binding and on Number of Platelets in Test Suspension*

Type of serum	Dilution of the serum	Trypsin 200 U	40 U	control
Normal human	1 2	270[a])	268	124
	1 8	286	270	161
ITP patient	1 2	603	518	310
	1 8	507	517	490
Rabbit-IC	1 20	828	967	752
Patelets		78	74	52
Total number of platelets		414	309	830
Number of aggregates		172	152	228

[a]) cps
Total counts added per test tube approx. 7900 cps
ITP, idiopathic thrombocytopenic purpura
Rabbit-IC, rabbit serum containing immune complexes formed in vivo

Acta path microbiol scand Sect. C, 86 237–244, 1978

IMMUNOCHEMISTRY OF *SALMONELLA* O-ANTIGENS:

Characterization of the Antibody Response to Tyvelose $1 \underset{\alpha}{\rightarrow} 3$ *Mannose* $1 \rightarrow$ *Bovine Serum Albumin Representative of* Salmonella *O-Antigen 9*

HANS ERIK CARLSSON and ALF A LINDBERG

Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden

Carlsson, H E. & Lindberg, A A. Immunochemistry of *Salmonella* O-antigens Characterization of the antibody response to tyvelose $1 \underset{\alpha}{\rightarrow} 3$ mannose $1 \rightarrow$ bovine serum albumin representative of *Salmonella* O-antigen 9 Acta path. microbiol scand Sect. C. 86 237–244, 1978

Immunization of rabbits with the synthetic disaccharide-protein conjugate, tyvelose $1 \underset{\alpha}{\rightarrow} 3$ mannose $1 \rightarrow$ bovine serum albumin (TM-BSA) in Freund's complete adjuvant, gave rise to antibodies directed against both the disaccharide hapten and the carrier protein The hapten antibodies were specific for O-antigen 9 of *Salmonella* and were superior in terms of specificity to conventional O factor 9 antibodies, as judged by passive haemagglutination and enzyme-linked immunosorbent assay (ELISA). A complement dependent bactericidal assay demonstrated that the TM-BSA antibodies had bacteriolytic activity also directed against the O-antigen 9 of *Salmonella* The high specificity was evident in both the IgG and IgM antibody classes In the bactericidal assay the IgM antibodies were more active than antibodies of the IgG class The results suggested that IgG antibodies in hyperimmune serum blocked the complement dependent bactericidal activity of IgM antibodies

Key words *Salmonella* O-antigens, immunochemistry, antibody response

A A Lindberg, Department of Bacteriology, National Bacteriological Laboratory, S-105 21, Stockholm, Sweden

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In studies of the immunochemistry of the O-antigen of *Salmonella* by *Lüderitz et al.* (1966), it was evident that the presence of dideoxyhexoses in serogroups A, B and D was of paramount importance for the group specificity of these O-antigens The concept immunodominant sugar was also coined However, attempts to use the dideoxysugar alone as haptenic group linked to an immunogenic carrier protein for production of antibacterial antibodies in rabbits failed (*Staub et al* 1966) The work of *Arakatsu et al.* (1966), where various maltose oligomers were used as haptens, demonstrated that a structure greater than a terminal α-glucopyranosyl unit was required to elicit precipitating antibodies. Subsequently, *Stellner et al* (1972) showed that the addition of an O-acetyl group to a hexose yielded a hapten which when coupled to bovine serum albumin (BSA) stimulated the formation of precipitating and agglutinating antibodies. From these results it may be assumed that a structure larger than one monosaccharide, coupled as hapten to an immunogenic carrier molecule, is needed to obtain an antibody response against the immunodominant part of a carbohydrate antigenic determinant. A carrier molecule substituted by a disaccharide would, therefore, be expected to give rise to antibodies against the disaccharide hapten.

The object of this investigation was to study the applicability of this hypothesis, using synthetic disaccharides representative of *Salmonella* O-antigens as haptens. A report is given of the specificity and biological activity of antibodies elicited in

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(b) *Inhibition studies* A suboptimal dilution of 0.5 ml of antiserum (giving an absorption of about 2 at 400 nm per 100 min in the final test) was added to an equal volume of different concentrations of the inhibitor in antigen coated tubes The tubes were incubated for 5 hours and washed, and sheep anti-rabbit Ig-alkaline phosphatase was added The assay was then performed as for titration of antibodies. The experiments were run in duplicate Maximum absorbance was determined in controls with PBS instead of inhibitor.

RESULTS

Sera collected from rabbits immunized with the tyvelose 1→3 mannose 1→ bovine serum albumin (TM-BSA) conjugate in Freund's complete adjuvant were assayed qualitatively as well as quantitatively (Tables 1 and 2).

Passive Haemagglutination Assay

Phenol-water extracted lipopolysaccharides (LPS) from *S typhimurium* LT2 (O-antigens 4, 5, 12_2) and *S. typhi* T2 (O9, 12_2) were used as antigens for coating sheep red blood cells. Already 10 days after immunization the rabbits had responded with a high agglutination titre (Table 1) The antibody activity was found exclusively in the IgM containing peak isolated after gel filtration of the sera on Sephadex G200. The reciprocal IgM titre remained unchanged after absorption of the serum with the *Staphylococcus aureus* Cowan 1 strain. Serum collected on day 40 (10 days after a booster injection) had the same, or slightly lower, agglutination titre. The TM-BSA antiserum diluted 1.5 did not react with the heterologous *S. typhimurium* LPS Nor did anti BSA control sera contain antibodies with specificity directed against the two LPS tested.

Enzyme Linked Immunosorbent Assay (ELISA)

The passive haemagglutination assay detects preferentially antibodies of the IgM class. The assay is, in fact, up to a hundred times more sensitive for detection of IgM than IgG antibodies ELISA detects these two antibody classes with approximately the same efficiency, but is in addition about a hundred times more sensitive. ELISA was therefore used for analysis of the antibody response to TM-BSA.

The reciprocal endpoint titre against the *S. typhi* T2 (O9, 12_2) antigen of sera collected on day 10 was 35 000, whereas a titre ≤ 100 (= preimmunization value), was seen against the *S. typhimurium*

TABLE 1 *Bactericidal, Passive Haemagglutination and ELISA Titres in Rabbits Immunized with TM-BSA Antigen*

Antiserum[a]	Reciprocal titres median values								
	Pre-immunization			Day 10			Day 40		
	Bactericidal[b]	PHA[c]	ELISA[d] × 10^{-3}	Bactericidal	PHA	ELISA × 10^{-3}	Bactericidal	PHA	ELISA × 10^{-3}
TM BSA	100	<10	<1	25 600	640	35	6400	160	1000
TM BSA-abs	<100	<10	<1	51 200	320		51 200	320	
IgG containing fraction				100	<10	<1	800	10	5000
IgG abs				<100	<10	<1	<100	<10	150
IgM containing fraction				25 600	320	27	12 800	160	20
IgM-abs				51 200	320	20	102 400	160	14
O factor 9	100	<10		819 200	640	5500			
BSA	100	<10		<100	<10		100	<10	

[a] Pools of antisera obtained from rabbits after immunization with either TM-BSA, BSA or heat-killed bacteria. O factor 9 serum was obtained after absorption according to *Kauffmann* (1966) The designation »-abs«, as in TM-BSA-abs, denotes that the serum was absorbed with the *Staphylococcus aureus* Cowan 1 strain which preferentially removes IgG antibodies

[b] Reciprocal of the dilution of pooled rabbit antisera causing 50% killing of *S enteritidis* Br '203bM in the presence of guinea-pig complement in 60 min at 37°C

[c] Median passive haemagglutination antibody titre determined with sheep red blood cells coated with phenol-water extracted LPS from *S typhi* T2 The titre is expressed as the reciprocal of the highest serum dilution giving haemagglutination visible to the naked eye

[d] Median ELISA titres were determined as endpoint titres using phenol-water extracted LPS from *S. typhi* T2 as antigen Coating dose = 1 μg LPS/ml

A blank space indicates that no titre determinations were made

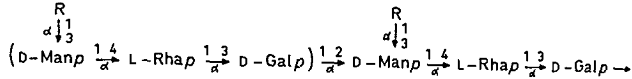


Fig. 1. Main structural features of the O-antigenic polysaccharide chain of *Salmonella* serogroup B and D bacteria In serogroup B, R indicates abequose (O antigen 4 specificity), in serogroup D, R indicates tyvelose (O antigen 9 specificity). The synthetic antigen is the phenylisothiocyanato disaccharide derivative covalently linked to BSA. The number of repeating units in O chain, varies from 4 to 30.
Abbreviations: Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Tyv, tyvelose.

rabbits immunized with tyvelose $1 \xrightarrow{\alpha} 3$ mannose $1 \rightarrow$ bovine serum albumin (TM-BSA) representative of O-antigen 9 of *Salmonella* serogroup D (Fig. 1).

MATERIALS AND METHODS

Bacterial strains. Salmonella typhimurium LT2 (O-antigen 4, 5 12_2) and *S. typhi* T2 (O9, 12_1, 12_3) were from the strain collection of the Department of Bacteriology. *S. typhimurium* P173C (O4, 5, 12_2) and *S. enteritidis* Br'203bM (O9, 12)were obtained form *R. J. Roantree,* Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif., USA. *Staphylococcus aureus* strain Cowan 1 was from the strain collection of this Department.

Lipopolysaccharide preparation The *Salmonella* bacteria were grown in complex broth medium as described previously (*Lindberg & Holme* 1972). Lipopolysaccharide (LPS) was extracted by phenol-water extraction from formaldehyde-killed bacteria (*Lindberg & Holme* 1972). The LPS preparations were spun in the ultracentrifuge (105 000 × *g*) until they were essentially free from material absorbing at 260 nm If necessary, any nucleic acid remaining was removed by treatment with ribonuclease (5 × Crystalline, Nutritional Biochemical Corp, Cleveland, Ohio, USA)

Disaccharide-protein conjugate preparation The disaccharide derivative [*p*-isothiocyanatophenyl-3-*O*-(3,6-dideoxy-α-*arabino*-hexopyranosyl)-α-D-mannopyranoside] was synthesized as described previously (*Ekborg et al.* 1975) and coupled to bovine serum albumin (BSA) as described by *McBroom et al* (1972). The average substitution of disaccharide was 20 mol per mol BSA

Immunization Procedures

(a) *Anti*-Salmonella *O-antigen sera.* Five male New Zealand white rabbits were immunized intravenously on days 1, 6 and 11; each injection contained approximately 1×10^{10} heat-killed bacteria. The rabbits were bled one week after the last injection Monospecific O factor 9 serum was prepared by absorption of pooled whole serum with heat-killed, washed and packed bacteria, according to *Kauffman* (1966).

(b) *Antiserum against disaccharide-protein conjugate* Three male New Zealand white rabbits were immunized in the popliteal lymph nodes with 10 μg of the synthetic antigen tyvelose $1 \xrightarrow{\alpha} 3$ mannose $1 \rightarrow$ BSA (TM-BSA) suspended in Freund's complete adjuvant. Each rabbit was immunized three times at four-weekly intervals and blood was collected before and 10 days after each injection. As control, three rabbits were also immunized with native BSA according to the same schedule as above.

Fractionation of serum. Rabbit serum was fractionated by gel filtration on Sephadex G200 (Pharmacia AB, Uppsala, Sweden) using 0.05 M phosphate-buffered saline (PBS), pH 7.2, as eluent. The IgM and IgG containing peak fractions were pooled and concentrated to the original serum volume

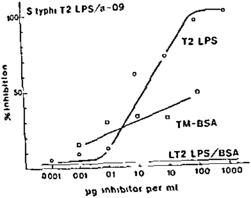
Absorption with Staphylococcus aureus *Cowan 1.* Staphylococci were prepared as described by *Jonsson & Kronvall* (1974). For absorption, equal volumes of a 1% (v/v) staphylococcal suspension and serum diluted 10^{-2} in PBS were mixed and kept at room temperature for 30 min. The tubes were spun at 1,500 *g* for 15 min, and the supernatant was removed for assays.

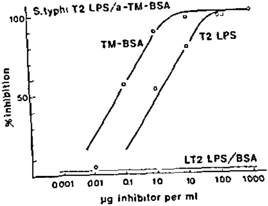
Passive haemagglutination assays Experiments were performed with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Virginia, USA) using 25μl loops and 1% sheep erythrocyte suspensions coated with phenol-water extracted LPS (*Lindberg & Holme* 1968) Agglutination was recorded after incubation at room temperature and in the cold (+ 4°C) for 1 and 18 h, respectively. All titrations were repeated in independent experiments

Bactericidal assay The partly rough, serum and complement sensitive strains *S enteritidis* Br '203bM and *S. typhimurium P173C* served as test bacteria The experiments were performed in duplicate with guinea-pig serum as complement source, as described previously (*Lindberg et al* 1974)

Enzyme-linked Immunosorbent Assay (ELISA)

(a) *Titration of antibodies.* ELISA was performed in duplicate according to *Engvall & Perlmann* (1972) as described previously (*Carlsson et al.* 1972) Antibodies against rabbit immunoglobulin were obtained by purification of hyperimmune sheep antiserum on insolubilized rabbit IgG. The anti-rabbit immunoglobulin was conjugated with alkaline phosphatase by the addition of glutardialdehyde. Conjugated material was separated from un-conjugated enzyme and immunoglobulin by gel filtration on Sepharose 6B (Pharmacia AB, Uppsala, Sweden).





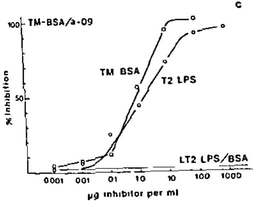


Fig 3 Quantification of antigen by means of inhibition of antibody binding to antigen coated tubes in ELISA. TM-BSA (□), BSA and LPS from *S. typhi* T2 (O) and *S typhimurium* LT2 were used as inhibitors in concentrations ranging from 0 001 to 1000 µg per ml in three different antigen-antibody systems.
(a) *S typhi* T2 LPS and O factor 9 antiserum,
(b) *S typhi* T2 LPS and TM-BSA antiserum and
(c) TM-BSA and O factor 9 antiserum.
The tubes were coated with 1 µg per ml LPS from *S typhi* T2 (a and b) or 0 1 µg per ml TM-BSA (c)
O factor 9 serum was used in dilution 10^{-4} and anti TM-BSA serum in dilution 2×10^{-5}.

respectively The opposite was true in sera collected on day 40, where the IgM containing fraction had an ELISA titre of 20 000 and the IgG containing fraction a titre of 5 000 000 (Table 1)

The IgG and IgM containing fractions were also absorbed with the *Staph aureus* Cowan 1 strain to remove IgG antibodies We found that whereas the ELISA titre of the IgM containing fraction was only slightly reduced, more than 97% of the IgG antibody titre against the *S typhi* T2 antigen was removed The reduction in titre seen in the IgM containing fraction might have been caused by absorption of aggregated IgG antibodies present in this fraction

Bactericidal Antibody Assays

The bactericidal complement dependent antibody activity of the TM-BSA antisera was estimated with guinea-pig serum as complement source, using the serum sensitive *S enteritidis* Br '203bM and *S typhimurium* P173C strains as indicator bacteria.

Serum collected on day 10 had a high bactericidal titre, viz. 25 600 against the *S enteritidis* strain, all of which was found in the IgM containing fraction. The bactericidal titre against the *S. typhimurium* strain was as low as that observed before immunization

In sera collected on day 40, the bactericidal titre against the *S enteritidis* strain appeared to be one-fourth of that seen in sera collected on day 10. Absorption of the pooled serum with the *Staph aureus* Cowan 1 strain resulted in an eight-fold increase in the reciprocal bactericidal titre to 51 200. Assays for the bactericidal activity of the IgG and IgM containing fractions before and after absorption with the Cowan 1 strain showed clearly that the bactericidal activity was to be found in the IgM containing fraction. The fact that the titre of the IgM containing fraction increased from 12 800 to 102 400 on absorption with the Cowan 1 strain was probably the result of the removal of aggregated IgG

The bactericidal activity of the O factor 9 antiserum was eight times higher than that of the TM-BSA serum. However, the specificity was not as high, since a low but significant activity against the *S. typhimurium* strain was demonstrated. No bactericidal activity above preimmunization level

TABLE 2. *Median Antibody Titre of Sera from Three Rabbits Immunized with TM-BSA Antigen*

Antigen[a]	ELISA end-point titre (× 10^{-3})			
	Day 0	Day 10	Day 40	Day 130
S. typhi T2	<1	35	3400	3000
S. typhimurium				
LT2	<1	<1	1	<1
BSA	<1	<1	100	300

[a] Tubes were coated with 1 μg of LPS or BSAper ml. The LPS was phenol-water extracted from *S. typhi* T2 or *S. typhimurium* LT2 bacteria.

LT2 (O4, 5, 12_2) antigen (Fig. 2, Table 1). Maximum titres were seen 10 days after the first booster injection (day 40), with a median value of 3 400 000. *Additional booster injections did not* enhance the antibody titre against *S. typhi* T2 antigen. The specificity of the antibody response was evident, since the titre against the *S. typhimurium* LT2 antigen was not more than 1000, i.e. a 5000-fold difference.

The difference in ELISA titre of an O factor 9 serum against the two antigens was less than 100-fold. Against *S. typhi* T2 LPS the titre was 55 000 000 and against *S. typhimurium* LT2 LPS 600 000.

Again, antiserum against native BSA did not react with the *S. typhi* T2 or *S. typhimurium* LT2 LPS preparations. The rabbits immunized with TM-BSA also responded with antibody formation against the BSA carrier protein. However, a high titre was not seen until after one booster injection. The titre observed (300 000) was one-tenth of that against the disaccharide hapten.

In order to determine the relative antibody activity of the immunoglobulin M and G classes, sera collected on days 10 and 40 were subjected to gel filtration on Sephadex G200. In sera collected on day 10 virtually all antibody activity was found in the IgM containing fraction, as opposed to the IgG containing fraction; the titres were 27 000 and 500.

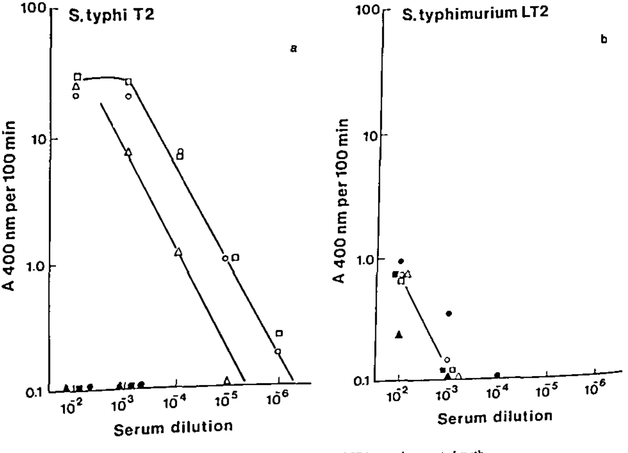
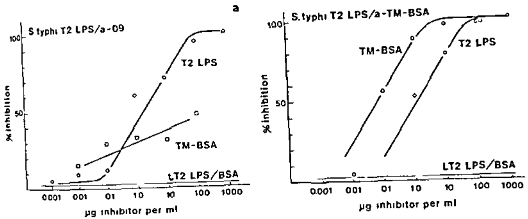


Fig 2. Titration of TM-BSA antisera from three rabbits by ELISA in tubes coated with
(a) 1 μg/ml of LPS from *S. typhi* T2;
(b) 1 μg/ml of LPS from *S. typhimurium* LT2.
Closed symbols: serum collected from rabbits before immunization
Open symbols: serum collected from rabbits on day 40, i.e. ten days after the booster immunization



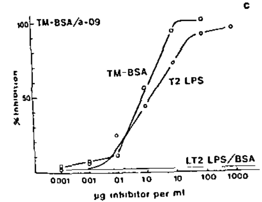


Fig 3 Quantification of antigen by means of inhibition of antibody binding to antigen coated tubes in ELISA TM-BSA (□), BSA and LPS from *S typhi* T2 (O) and *S. typhimurium* LT2 were used as inhibitors in concentrations ranging from 0 001 to 1000 μg per ml in three different antigen-antibody systems
(a) *S typhi* T2 LPS and O factor 9 antiserum,
(b) *S typhi* T2 LPS and TM-BSA antiserum and
(c) TM-BSA and O factor 9 antiserum
The tubes were coated with 1 μg per ml LPS from *S typhi* T2 (a and b) or 0 1 μg per ml TM-BSA (c) O factor 9 serum was used in dilution 10^{-6} and anti TM-BSA serum in dilution 2×10^{-5}.

respectively The opposite was true in sera collected on day 40, where the IgM containing fraction had an ELISA titre of 20 000 and the IgG containing fraction a titre of 5 000 000 (Table 1).

The IgG and IgM containing fractions were also absorbed with the *Staph aureus* Cowan 1 strain to remove IgG antibodies We found that whereas the ELISA titre of the IgM containing fraction was only slightly reduced, more than 97% of the IgG antibody titre against the *S typhi* T2 antigen was removed The reduction in titre seen in the IgM containing fraction might have been caused by absorption of aggregated IgG antibodies present in this fraction

Bactericidal Antibody Assays

The bactericidal complement dependent antibody activity of the TM-BSA antisera was estimated with guinea-pig serum as complement source, using the serum sensitive *S enteritidis* Br '203bM and *S. typhimurium* P173C strains as indicator bacteria.

Serum collected on day 10 had a high bactericidal titre, viz. 25 600 against the *S. enteritidis* strain, all of which was found in the IgM containing fraction. The bactericidal titre against the *S typhimurium* strain was as low as that observed before immunization

In sera collected on day 40, the bactericidal titre against the *S. enteritidis* strain appeared to be one-fourth of that seen in sera collected on day 10. Absorption of the pooled serum with the *Staph. aureus* Cowan 1 strain resulted in an eight-fold increase in the reciprocal bactericidal titre to 51 200. Assays for the bactericidal activity of the IgG and IgM containing fractions before and after absorption with the Cowan 1 strain showed clearly that the bactericidal activity was to be found in the IgM containing fraction. The fact that the titre of the IgM containing fraction increased from 12 800 to 102 400 on absorption with the Cowan 1 strain was probably the result of the removal of aggregated IgG.

The bactericidal activity of the O factor 9 antiserum was eight times higher than that of the TM-BSA serum. However, the specificity was not as high, since a low but significant activity against the *S typhimurium* strain was demonstrated. No bactericidal activity above preimmunization level

TABLE 2. *Median Antibody Titre of Sera from Three Rabbits Immunized with TM-BSA Antigen*

Antigen[a]	ELISA end-point titre ($\times 10^{-3}$)			
	Day 0	Day 10	Day 40	Day 130
S. typhi T2	<1	35	3400	3000
S. typhimurium LT2	<1	<1	1	<1
BSA	<1	<1	100	300

[a] Tubes were coated with 1 μg of LPS or BSAper ml. The LPS was phenol-water extracted from *S. typhi* T2 or *S. typhimurium* LT2 bacteria.

LT2 (O4, 5, 12_2) antigen (Fig. 2, Table 1). Maximum titres were seen 10 days after the first booster injection (day 40), with a median value of 3 400 000. Additional booster injections did not enhance the antibody titre against *S. typhi* T2 antigen. The specificity of the antibody response was evident, since the titre against the *S typhimurium* LT2 antigen was not more than 1000, i.e. a 5000-fold difference.

The difference in ELISA titre of an O factor 9 serum against the two antigens was less than 100-fold. Against *S. typhi* T2 LPS the titre was 55 000 000 and against *S. typhimurium* LT2 LPS 600 000.

Again, antiserum against native BSA did not react with the *S. typhi* T2 or *S. typhimurium* LT2 LPS preparations. The rabbits immunized with TM-BSA also responded with antibody formation against the BSA carrier protein. However, a high titre was not seen until after one booster injection The titre observed (300 000) was one-tenth of that against the disaccharide hapten.

In order to determine the relative antibody activity of the immunoglobulin M and G classes, sera collected on days 10 and 40 were subjected to gel filtration on Sephadex G200. In sera collected on day 10 virtually all antibody activity was found in the IgM containing fraction, as opposed to the IgG containing fraction; the titres were 27 000 and 500,

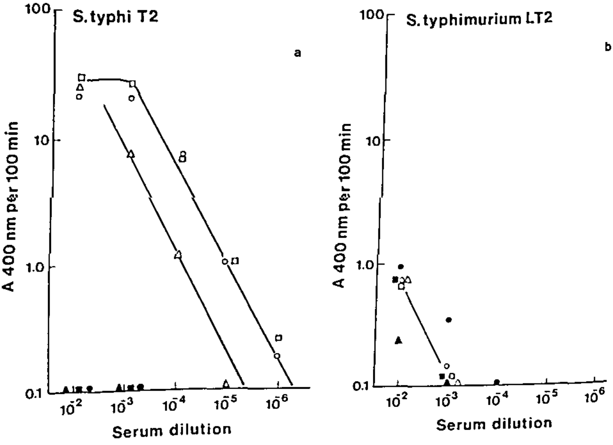
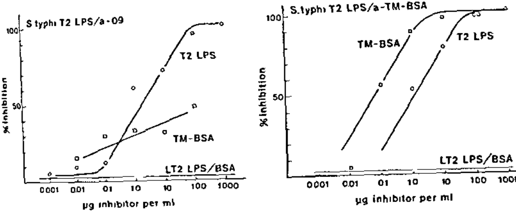


Fig. 2. Titration of TM-BSA antisera from three rabbits by ELISA in tubes coated with
(a) 1 μg/ml of LPS from *S. typhi* T2;
(b) 1 μg/ml of LPS from *S. typhimurium* LT2.
Closed symbols: serum collected from rabbits before immunization
Open symbols: serum collected from rabbits on day 40, i.e. ten days after the booster immunization



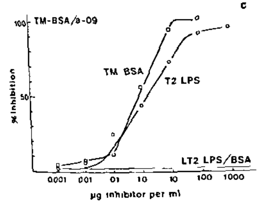


Fig 3 Quantification of antigen by means of inhibition of antibody binding to antigen coated tubes in ELISA TM-BSA (□), BSA and LPS from *S. typhi* T2 (O) and *S typhimurium* LT2 were used as inhibitors in concentrations ranging from 0 001 to 1000 µg per ml in three different antigen-antibody systems
(a) *S typhi* T2 LPS and O factor 9 antiserum,
(b) *S typhi* T2 LPS and TM-BSA antiserum and
(c) TM-BSA and O factor 9 antiserum
The tubes were coated with 1 µg per ml LPS from *S typhi* T2 (a and b) or 0 1 µg per ml TM-BSA (c)
O factor 9 serum was used in dilution 10^{-6} and anti TM-BSA serum in dilution 2×10^{-5}

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was observed against *S. enteritidis* or *S. typhimurium* in sera from rabbits immunized with native BSA.

Inhibition Studies Using ELISA

The specificity of the TM-BSA antiserum was assayed further in an inhibition system and compared with that of O factor 9 serum (Fig. 3). Three different inhibition systems were used (i) *S. typhi* T2 LPS as antigen with O factor 9 antiserum, (ii) *S. typhi* T2 LPS as antigen with TM-BSA antiserum, and (iii) TM-BSA as antigen with O factor 9 antiserum.

The concentration of *S. typhi* T2 LPS which caused a 50% inhibition of the *S. typhi* T2 LPS/O factor 9 antiserum system (Fig. 3a) was 1.0 μg per ml. For TM-BSA the concentration was more than a hundred times higher. Furthermore, the slope of the inhibition curve was significantly different. Neither *S. typhimurium* LT2 LPS nor BSA caused any inhibition in the highest concentration tested (1000 μg). In the system with TM-BSA as antigen and O factor 9 antiserum (Fig. 3c), the *S.typhi* T2 LPS was as effective an inhibitor as the disaccharide-protein conjugate. The concentrations were approximately 1.0 μg per ml. Again neither the *S. typhimurium* LT2 LPS nor the native BSA caused any measurable inhibition.

Use of *S.typhi* T2 LPS as antigen in the system with TM-BSA antiserum (Fig. 3b) revealed that, under these circumstances, the TM-BSA conjugate was the most potent inhibitor. Approximately 0.05 μg per ml of TM-BSA caused a 50% inhibition, whereas 1.0 μg per ml of the *S. typhi* T2 LPS was required. Again no inhibition was observed with the highest concentrations of *S. typhimurium* LT2 LPS and BSA.

DISCUSSION

The synthetic disaccharide-protein conjugate, tyvelose 1 $\overrightarrow{a}$ 3 mannose 1 → BSA, suspended in Freund's complete adjuvant readily stimulated production of humoral antibodies with specificity for both the haptenic group and the carrier protein (Table 2).

The specificity of the antibodies directed against the disaccharide was high, considerably higher than a conventional O factor 9 antiserum. This was evident in the ELISA, where LPS from *Salmonella* serogroups D and B were used as antigens. The ratio homologous/heterologous titre for the TM-BSA antiserum was in the range 5000:1 (Table 2, Fig. 2). For the O factor 9 serum the corresponding figure was approximately 100:1. Indirect immunofluorescence studies, using anti TM-BSA serum on 975 enteric bacterial strains other than *Salmonella* serogroup D, showed no positive fluorescence reactions with these bacteria, except in eight cases (seven *Bacteroides fragilis* and one *Yersinia pseudotuberculosis*) (*Svenungsson & Lindberg* 1977). These results corroborate the specificity observed in the present investigation.

The ELISA inhibition systems used confirmed the specificity of the TM-BSA antiserum seen in antibody titrations. However, there were differences in the inhibiting activity of the *S. typhi* T2 LPS and TM-BSA antigens. Thus the *S. typhi* T2 LPS was a hundred times better inhibitor than TM-BSA in the O factor 9/*S. typhi* T2 LPS system (Fig. 3a), whereas TM- BSA was twenty times more active in the TM-BSA antiserum/*S. typhi* T2 LPS system (Fig. 3b). These findings were in accordance with the results of quantitative precipitin inhibition assays (*Jörbeck et al.* 1978). The differences observed are probably the result of differences in shape and size of the antibody combining sites. The O factor 9 antibody is elicited by stimulation with an antigenic determinant where the D-mannose residue of the disaccharide is linked to D-galactose and L-rhamnose (Fig. 1), whereas for the TM-BSA antibodies the D-mannose residue is linked via a *p*-isothiocyanatophenyl group to the ε amino group of lysine in BSA. It can be surmised that both the linkage group and the lysine residue participate in forming the combining site with specificity for the disaccharide hapten. A similar participation was demonstrated with conjugated $(D\text{-}Ala)_n$ Gly haptens smaller than a tetrapeptide (*Schechter et al.* 1970).

The high specificity of the TM-BSA antiserum as compared to the O factor 9 serum was also evident in the passive haemagglutination and the complement-dependent bactericidal assays.

The complement-dependent bactericidal activity of the TM-BSA antibodies (Table 1) is of interest. Thus, the antibodies elicited against the synthetic disaccharide have the same biological activity as antibodies elicited by heat-killed bacteria. The highest titre in whole serum was seen 10 days after the primary immunization (Table 1). The bactericidal titre of serum collected 10 days after a booster immunization was sixteen times lower. However, removal of IgG antibodies from serum by absorption with a protein A containing *Staph. aureus* strain revealed that the bactericidal titre of the hyperimmune serum was, in fact, as high as in that collected 10 days after the primary immunization. Absorption with protein A from *Staph. aureus* preferentially removes antibodies of the IgG class (*Kronvall & Williams* 1969; *Kronvall et al.* 1970). This suggests that the IgG antibodies in the hyperimmune serum blocked the bactericidal activity of the IgM antibodies.

Subsequent Sephadex G200 gel filtration of sera collected on days 10 and 40 and separation into IgM and IgG containing fractions demonstrated that most of the complement dependent bactericidal activity was to be found in the IgM containing fraction This is in agreement with earlier observations (*Robbins et al.* 1965) Absorptions of the IgG and IgM containing fractions with *Staph aureus* Cowan 1 eliminated the bactericidal activity of the IgG containing fraction. Thus also the IgG antibodies were bacteriolytic in the presence of complement, although not as active as IgM antibodies In the IgM containing fraction from the serum pool obtained on day 40, an eight-fold increase in activity was seen after absorption. This was probably caused by removal of aggregated IgG from the IgM containing fraction. Thus, the results support further the hypothesis that IgG antibodies can block the bactericidal activity of IgM antibodies

Hall et al. (1971) observed that IgA antibodies blocked the bactericidal activity of IgG and IgM antibodies Our fractionation does not exclude that the IgM containing fraction could contain IgA However, the concentration of serum IgA does not seem to increase during parenteral immunization (*Cebra & Robbins* 1966) Accordingly, unless there was a marked shift in antibody specificity within the IgA fraction during the interval between 10 and 40 days when sera were collected, the blocking effect seen after 40 days should have been seen also after 10 days It is also unknown whether or not absorptions of serum with protein A remove rabbit IgA.

It is evident that the use of the tyvelose $1 \xrightarrow{\alpha} 3$ mannose disaccharide as hapten linked to BSA as immunogenic carrier has resulted in an immunogen which elicits in rabbits antibodies with a higher specificity for *Salmonella* O-antigen 9 than those obtained after conventional immunization and absorption procedures Such antibody preparations form a basis for a more accurate diagnosis of *Salmonella* infections The superiority of the TM-BSA antiserum over O factor 9 serum for identification of *Salmonella* serogroup D bacteria by indirect immunofluorescence has already been proved (*Svenungsson & Lindberg* 1977). Moreover, the ELISA inhibition assay opens possibilities for detection of *Salmonella* O-antigen in the nano- to microgram range, either soluble or particulate, in biological material without prior enrichment by cultivation

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THE PREVALENCE OF IgE ANTINUCLEAR ANTIBODIES IN RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS

HENRIK PERMIN and ALLAN WIIK

Laboratory of Immunology, University Clinic for Infectious Diseases, Rigshospitalet, Copenhagen, Denmark

Permin, H & Wiik, A The prevalence of IgE antinuclear antibodies in rheumatoid arthritis and systemic lupus erythematosus Acta path microbiol scand Sect. C, 86 245–249, 1978

Antinuclear antibodies (ANA) of the IgE class were studied in sera from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and healthy controls. Sixty per cent of 20 RA patients with neutropenia were found to have IgE granulocyte-specific (GS-) ANA, whereas only 16% of RA patients without neutropenia had IgE antibodies of similar specificity About 5% in each group of RA patients had IgE organ-nonspecific (ON-) ANA Eleven of 15 patients with active SLE and only 4 of 20 with inactive SLE had IgE ON-ANA Sera from five patients with lupus nephritis all contained IgE ON-ANA None of 100 sera from controls showed presence of IgE ANA. IgE ANA titres in RA and SLE patients correlated to the titres of ANA of the other four immunoglobulin classes Gel filtration studies at neutral and acid pH of RA sera containing high titres of IgE GS-ANA indicated the presence of these antibodies in immune complexes Studies of serum cryoprecipitates supported this conclusion IgE ANA production may be of pathogenetic importance in RA and SLE by eliciting type-I reactions

Key words Antinuclear antibodies, granulocyte-specific; IgE, rheumatoid arthritis, neutropenia, systemic lupus erythematosus, disease activity.

H Permin, Laboratory of Immunology, Department M, Rigshospitalet, DK-2200 Copenhagen N, Denmark

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Immunoglobulins of the E class (IgE) have been demonstrated in the skin and the blood of individuals with atopic diseases These immunoglobulins have been shown to form the majority of the so-called reagenic antibodies (13) They closely resemble other immunoglobulins, but have certain physicochemical and physiological properties of their own, especially the tissue binding ability (13) The physiological function of IgE has long been obscure but recent studies suggest that IgE may play a role in the total immunological homeostasis as well as a specific role in the defence against parasites (3, 13)

IgE class antinuclear antibodies (ANA) have been described in systemic lupus erythematosus (SLE) (14, 16), rheumatoid arthritis (RA) (16, 17, 18), and psoriasis (5), and IgE has been found deposited in human tissues such as thyroid and kidney in disease (13, 15, 20). These findings suggest that IgE may play a role in autoimmunity (3, 18)

The present study was undertaken to investigate the occurrence and titres of granulocyte-specific and organ-nonspecific (GS- and ON-)ANA of the IgE class in RA and SLE, and to establish the possible relation of these antibodies to clinical activity in these diseases

MATERIALS AND METHODS

Eighty-four patients had classical or definite RA by the American Rheumatism Association criteria (19) Twenty RA patients had neutropenia (neutrophil counts $< 2 \times 10^9/l$), 16 were women and 4 were men, and the

average age was 63.9 years (range 42–82). Sixty-four patients had RA without neutropenia, 52 being women and 12 men, and the average age was 64.2 years (range 28–81). The neutropenic cases were selected from a large number of RA patients on the basis of neutropenia, while the non-neutropenic cases were ramdomly selected from consecutive RA patients studied serologically in our laboratory.

Thirty-five patients had SLE using the preliminary criteria of the American Rheumatism Association (4) Disease activity was estimated based on at least one of the following manifestations renal impairment, anaemia (Hb < 6 mmol/l), leukopenia (< 2 × 10^9/l), thrombocytopenia (platelet counts < 50 × 10^9/l), or neurological symptoms. The female/male ratio was 32/3, and the average age was 45.1 years (range 13–79). Fifteen patients had active disease, and 5 had lupus nephritis as verified by renal biopsy. Both groups of patients were randomly selected. Most patients in the groups of RA and SLE patients attended rheumatology and/or hematology departments in or around Copenhagen.

One hundred sera were drawn from healthy controls, 80 women and 20 men with an average age of 54.9 years (range 13–82).

Sera were investigated for the occurence of GS- and ON-ANA of the IgG, IgM, IgA, IgD and IgE classes as previously described (17, 22 – 25). Positive samples were titrated in two-fold dilutions using phosphate buffered saline pH 7.4 (PBS) as diluent. Fluorescein isothiocyanate (FITC)-labelled rabbit IgG specific for human γ, μ and α chains were purchased from Dakopatts, Denmark. Rabbit antisera specific for δ and ε chains were obtained from Behringwerke, GFR. The IgG fraction was isolated from each antiserum by DEAE fractionation and labelled with FITC as described previously (23) The antisera to γ, μ, α and δ showed monospecific reactions with normal human serum in crossed immunoelectrophoresis FITC-labelled antisera were tested for specificity by means of IgG, IgM, IgA and IgD monoclonal bone marrow specimens from patients with multiple myeloma and macroglobulinaemia, and on a continuously cultured IgE producing myeloma cell line (kindly donated by dr. *K. Nilsson*, Uppsala) (23, 24). The fluorescein/protein ratios estimated by the optical density ratio 495/280 nm were 0.5–0.7. The slides were examined in a Leitz Orthoplan fluorescence microscope equipped for incident light illumination using an Osram 200 mercury lamp as light source (22) GS- and ON-ANA have been defined in detail previously (17). Rheumatoid factors were demonstrated by the FII latex fixation slide test (Behringwerke) Titres ≥ 32 were considered abnormal

Three blood samples, from patients with RA exhibiting neutropenia, were clotted at 37°C for one hour, cells were removed by centrifugation at 400 *g* for 10 minutes and the sera were incubated at 4°C for one week The cryoprecipitable material was pelleted at 1000 *g* for 10 minutes, resuspended and washed 4 times in ice cold PBS in an attempt to remove nonspecifically trapped proteins from the precipitate. The cryoprecipitate was redissolved in a small volume of PBS at 37°C and insoluble material was removed by centrifugation at 1000 *g*.

Two RA sera containing high titres of IgE GS-ANA were both fractionated on a G-200 Sephadex column at pH = 7.35 using Tris-HCl buffer, ι = 0.154, and at pH = 4.50 using acetate buffer, i = 0.154, respectively, as described in detail earlier (21, 25)

Statistics

The statistical significance of the data obtained was evaluated by the Fisher's four-fold table test, Spearman's rank correlation test and chi-square test (6) Significance level 5%.

RESULTS

IgE ANA were found in 32% of the 84 sera from patients with RA. Most of the positive results were due to the high frequency of IgE GS-ANA (Table I) Sera from 20 patients with RA accompanied by neutropenia showed presence of IgE GS-ANA in 60%, whereas only 16% of RA patients without neutropenia were found to have this antibody ($p < 0.01$). The frequency of IgE ON-ANA was low in both groups.

TABLE I *The Prevalence of IgE GS- and ON-ANA in Patients with RA, SLE and Controls*

Subjects		IgE ANA	IgE GS-ANA	IgE ON-ANA
RA with neutropenia	(n = 20)	13 (65%)	12 (60%)[a]	1 (5%)
RA without neutropenia	(n = 64)	14 (22%)	10 (16%)[a]	4 (6%)
SLE with active disease	(n = 15)	11 (71%)	1 (7%)	11 (71%)[b]
SLE without active disease	(n = 20)	4 (20%)	0	4 (20%)[b]
Controls	(n = 100)	0	0	0

[a]) and [b]), $p < 0.01$.

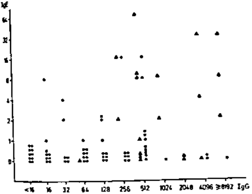


Fig 1 Relation between the titres of IgG and IgE GS-ANA in sera from patients with RA.
r = 0 26, p < 0 05
▲ = patients with neutropenia,
● = patients without neutropenia

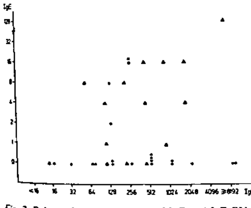


Fig 2 Relation between the titres of IgG and IgE ON-ANA in sera from patients with SLE
r = 0 50, p < 0 01
▲ = patients with active disease
● = patients with inactive disease

In contrast, only one serum from 35 patients with SLE contained IgE GS-ANA, while about half of the patients had IgE ON-ANA (Table 1) Among the 15 SLE patients with active disease 11 showed presence of IgE ON-ANA, compared to only 4 of 20 SLE patients with inactive disease ($p < 0\ 01$) Five patients with nephritis all had IgE ON-ANA The highest titre was found in a 29 year old female with pronounced disease activity (nephritis, serositis and high anti-DNA antibodies) None of the 100 control sera showed IgE ANA activity

A correlation of IgE ANA titres with those of IgG, IgM, IgA and IgD ANA was found when compared by Spearman's rank correlation test (p value in all cases $< 0\ 05$) Fig 1 and 2 show the titres of IgE and IgG ANA in RA and SLE patients, respectively, illustrating that high titres of IgG ANA in the absence of IgE ANA and vice versa was seen in several sera.

Three cryoprecipitates, from neutropenic RA patients having high titres of serum IgE GS-ANA, were found to contain IgE GS-ANA. Sera from two patients with high titres of IgE GS-ANA were fractionated by Sephadex G-200 gel filtration at neutral pH (Fig. 3) IgE GS-ANA was detected both in the void volume fractions and the 8 S containing fractions of the second peak. The same sera were fractionated also by Sephadex G-200 gel filtration at pH 4.50 (Fig 4), but now all IgE GS-ANA activity was contained in the second peak fractions, most probably due to dissociation of immune complexes involving IgE GS-ANA (9, 21, 25).

Sixteen of the 20 RA patients with neutropenia had positive latex tests in titres up to 1024, while only 31 of 64 non-neutropenic RA patients had positive latex tests and titres up to 256. Thirteen SLE patients had positive latex tests in titres up to 512, and no difference was found between patients with active and inactive disease. No covariation was seen between IgE ANA and rheumatoid factors

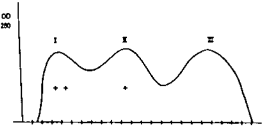


Fig 3 Three ml of serum from a patient with rheumatoid arthritis and accompanying neutropenia subjected to Sephadex G-200 gel filtration at pH 7 35 + designates IgE GS-ANA activity in the individual fractions obtained
Abscissa Fraction no

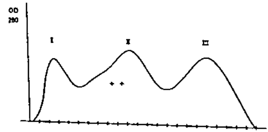


Fig 4 Three ml of the same serum subjected to Sephadex G-200 gel filtration at pH 4.50 + designates IgE GS-ANA activity in the individual fractions
Abscissa Fraction no.

average age was 63.9 years (range 42–82). Sixty-four patients had RA without neutropenia, 52 being women and 12 men, and the average age was 64.2 years (range 28–81) The neutropenic cases were selected from a large number of RA patients on the basis of neutropenia, while the non-neutropenic cases were ramdomly selected from consecutive RA patients studied serologically in our laboratory.

Thirty-five patients had SLE using the preliminary criteria of the American Rheumatism Association (4). Disease activity was estimated based on at least one of the following manifestations, renal impairment, anaemia (Hb < 6 mmol/l), leukopenia ($< 2 \times 10^9$/l), thrombocytopenia (platelet counts $< 50 \times 10^9$/l), or neurological symptoms. The female/male ratio was 32/3, and the average age was 45.1 years (range 13–79). Fifteen patients had active disease, and 5 had lupus nephritis as verified by renal biopsy. Both groups of patients were randomly selected. Most patients in the groups of RA and SLE patients attended rheumatology and/or hematology departments in or around Copenhagen

One hundred sera were drawn from healthy controls, 80 women and 20 men with an average age of 54.9 years (range 13–82)

Sera were investigated for the occurence of GS- and ON-ANA of the IgG, IgM, IgA, IgD and IgE classes as previously described (17, 22 – 25) Positive samples were titrated in two-fold dilutions using phosphate buffered saline pH 7.4 (PBS) as diluent. Fluorescein isothiocyanate (FITC)-labelled rabbit IgG specific for human γ, μ and α chains were purchased from Dakopatts, Denmark Rabbit antisera specific for δ and ε chains were obtained from Behringwerke, GFR The IgG fraction was isolated from each antiserum by DEAE fractionation and labelled with FITC as described previously (23) The antisera to γ, μ, α and δ showed monospecific reactions with normal human serum in crossed immunoelectrophoresis. FITC-labelled antisera were tested for specificity by means of IgG, IgM, IgA and IgD monoclonal bone marrow specimens from patients with multiple myeloma and macroglobulinaemia, and on a continuously cultured IgE producing myeloma cell line (kindly donated by dr. K Nilsson, Uppsala) (23, 24). The fluorescein/protein ratios estimated by the optical density ratio 495/280 nm were 0.5–0.7. The slides were examined in a Leitz Orthoplan fluorescence microscope equipped for incident light illumination using an Osram 200 mercury lamp as light source (22). GS- and ON-ANA have been defined in detail previously (17). Rheumatoid factors were demonstrated by the FII latex fixation slide test (Behringwerke) Titres ≥ 32 were considered abnormal

Three blood samples, from patients with RA exhibiting neutropenia, were clotted at 37°C for one hour, cells were removed by centrifugation at 400 *g* for 10 minutes and the sera were incubated at 4°C for one week. The cryoprecipitable material was pelleted at 1000 *g* for 10 minutes, resuspended and washed 4 times in ice cold PBS in an attempt to remove nonspecifically trapped proteins from the precipitate The cryoprecipitate was redissolved in a small volume of PBS at 37°C and insoluble material was removed by centrifugation at 1000 *g*

Two RA sera containing high titres of IgE GS-ANA were both fractionated on a G-200 Sephadex column at pH = 7.35 using Tris-HCl buffer, ι = 0.154, and at pH = 4.50 using acetate buffer, ι = 0 154, respectively, as described in detail earlier (21, 25)

Statistics

The statistical significance of the data obtained was evaluated by the Fisher's four-fold table test, Spearman's rank correlation test and chi-square test (6) Significance level 5%.

RESULTS

IgE ANA were found in 32% of the 84 sera from patients with RA. Most of the positive results were due to the high frequency of IgE GS-ANA (Table 1). Sera from 20 patients with RA accompanied by neutropenia showed presence of IgE GS-ANA in 60%, whereas only 16% of RA patients without neutropenia were found to have this antibody ($p < 0.01$). The frequency of IgE ON-ANA was low in both groups.

TABLE 1 *The Prevalence of IgE GS- and ON-ANA in Patients with RA, SLE and Controls*

Subjects		IgE ANA	IgE GS-ANA	IgE ON-ANA
RA with neutropenia	(n = 20)	13 (65%)	12 (60%)[a]	1 (5%)
RA without neutropenia	(n = 64)	14 (22%)	10 (16%)[a]	4 (6%)
SLE with active disease	(n = 15)	11 (71%)	1 (7%)	11 (71%)[b]
SLE without active disease	(n = 20)	4 (20%)	0	4 (20%)[b]
Controls	(n = 100)	0	0	0

[a]) and [b]). $p < 0.01$.

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DISCUSSION

The present study shows a higher prevalence of IgE GS-ANA in the neutropenic than in non-neutropenic cases of RA. Such neutropenic RA patients have pronounced serological activity as judged by presence in serum of high titres of IgG and IgM GS-ANA (24, 25), high titres of rheumatoid factors (1), circulating immune complexes (12, 21) and hypocomplementaemia (11).

The specificity of ANA in RA patients was predominantly directed against nuclei of granulocytes, whereas ANA in SLE patients was organnonspecific in agreement with former studies (7). The higher prevalence of IgE ON-ANA in SLE patients with active than with inactive disease confirm the results obtained by *Jackson et al.* (14).

In most of the cases in which IgE ANA activity was demonstrated antibodies with similar specificity were also detected in the IgG, IgM, IgA and IgD classes. This finding suggests that IgE ANA production is related to that of the 4 other immunoglobulin classes of ANA, most probably being part of a broad polyclonal antibody response to nuclear materials. The existence of a number of sera containing high titres of IgG, IgM, IgA or IgD ANA in the absence of IgE ANA and vice versa makes unwanted cross-reactivity of the different anti-immunoglobulin conjugates an unlikely explanation for the observed covariation.

It has been postulated that IgE in serum may be involved in immune complex formation (2, 3) This notion is supported by our finding of IgE GS-ANA in cryoprecipitates and in acid dissociable aggregates in neutropenic RA sera. A possible role of IgE in the pathophysiology of autoimmune diseases may be that of IgE mediated vasoactive amine release resulting in increased vascular permeability and hence enhancement of immune complex deposition (3). Vasoactive amine release might be mediated through autoallergic reactions between IgE ANA and nuclear antigens on the surface of basophils as has been suggested from recent observations in RA patients, the peripheral blood leukocytes of whom released histamine upon challenge with RNA and DNA (18). The production of IgE ANA as part of a broad polyclonal response to nuclear antigens might lead to type-I reactions and possibly explain the increased IgE levels and the eosinophilia found in some patients with RA (10), as well as the presence of allergic manifestations in these and in SLE patients (8, 18).

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EVIDENCE FOR NON-SPECIFICALLY BOUND IgG IN HUMAN TUMOURS

FINN WESENBERG and OLAV TÖNDER

Broegelmann Research Laboratory for Microbiology; and Department of Microbiology, The Gade Institute, University of Bergen, Bergen, Norway

Wesenberg, F & Tonder, O Evidence for non specifically bound IgG in human tumours Acta path microbiol scand Sect C, 86 251–258, 1978

Heat eluates of homogenized human malignant and normal tissues were prepared using the continuous flow elution technique IgG antibodies to rabbit erythrocytes served as marker antibodies for non-specifically bound IgG Eluates of all of the 13 malignant tumours tested contained IgG Most were eluted at 56° C, but considerable amounts also at 37° C and 45° C. Marker antibodies were present in eluates of 4 of the tumours, indicating that at least parts of the IgG associated with malignant human tumours are non-specifically bound. Eight tumours contained too little IgG to detect marker antibodies, and one contained high amounts of IgG without marker antibodies Eluates of normal organs contained either no or only small amounts of IgG, although apparently normal kidneys from older individuals contained some more IgG Marker antibodies were detected in some of the eluates indicating non-specific binding

Key words Tumour-associated IgG, non-specifically bound IgG

F Wesenberg, Broegelmann Research Laboratory for Microbiology, MFH-bygget, N-5016 Haukeland sykehus, Norway

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Reports published during the last decade have furnished evidence for the existence of antibodies directed towards tumour-specific antigens both in animals (5, 16) and humans (3, 5, 6, 10, 16). After the discovery of the Fc-receptors and their relationship to human malignancy (9, 12, 14), the concept of tumour-associated immunoglobulins was extended also to include non-specifically bound IgG *Braslawsky et al* (1) have shown that unrelated antibodies could bind to a non-lymphoid murine tumour In human malignancy conclusive evidence has not been provided for non-specific or Fc-bound immunoglobulins, although one report has indicated this (14)

Elution of antibodies from tissue specimens can be achieved by several techniques. Acid buffers, alkaline buffers, high ionic strength, chaotropic agents, temperatures between + 4° C and 56° C or combinations of these factors (4, p 781–789) have been used We describe here a technique using a column with a continuous flow of saline at 56° C which has proved to be an efficient method for the elution of immunoglobulins.

In a previous report (15) we have described that agglutinins to rabbit erythrocytes in extracts of human malignant and normal tissue are identical to the naturally occurring serum IgG antibody to rabbit erythrocytes. We have now extended this observation and have found that the ratio between titre of these antibodies to concentration of IgG is constant when serum and extracts of several organs from the same person are used. By comparing this ratio in extracts and eluates of human malignant tumours, non-specifically bound IgG was looked for.

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FINN WESENBERG and OLAV TÖNDER

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g for 10 min (100 per cent). Formalinized erythrocytes (4, p 22, alternative method) were prepared for some experiments

Haemagglutination in Tubes

To twofold dilutions of extracts, human IgG or eluates in 0 1 ml volumes of PBS, 0.1 ml of a 0 5 per cent suspension of rabbit erythrocytes was added. The mixtures were left for 15 min at ambient temperature. The tubes were centrifuged at 1000 x g for 30 s. The erythrocytes were gently resuspended and agglutination was recorded macroscopically. The titre of agglutination was defined as the reciprocal value of the highest dilution showing agglutination. One agglutinating unit was defined as the amount of the highest dilution of human IgG or serum which agglutinated an equal amount of a 0 5 per cent suspension of erythrocytes

After the direct agglutination was recorded, the erythrocytes in each tube were washed twice in 2 ml of PBS The erythrocytes were then resuspended in 0 1 ml of antiserum to human immunoglobulins diluted to 1/40th the anti-globulin titre dilution The tubes were left at ambient temperature for 5 min, centrifuged at 1000 x g for 30 s and the agglutination (indirect agglutination) was recorded as described above

All the titres given for extracts and eluates are indirect agglutination titres

Various Reagents

Glycine HCl buffer, pH 2 0, 3 M NaCl and 3 M NaSCN were standard reagents DNase (lot 71945) was purchased from Calbiochem, Los Angeles, USA, and collagenase (no C-6885) was purchased from Sigma Chemical Company, St Louis, Mo, USA

Immunofluorescent Test

2 drops of a 1 per cent erythrocyte suspension were incubated with an equal volume of FITC-conjugated antiserum at ambient temperature for 30 min. The erythrocytes were then washed 3 times in PBS at ambient temperature and mounted in 50 per cent glycerol in PBS They were examined for immunofluorescence using a Leitz Orthoplan Microscope equipped for epillumination with a Leitz vertical illuminator.

Quantitation of IgG

IgG was quantified using single radial immunodiffusion with commercial agar plates, purchased from Behringwerke AG, Marburg-Lahn, W-Germany.

RESULTS

Elution Procedure. Technical Aspects

Formalinized rabbit erythrocytes coated with 1/4 agglutinating unit of human natural antibodies or sheep erythrocytes coated with 1/4 agglutinating unit of rabbit immune antibodies served as control for dissociation of known antigen-antibody complexes. The recovery of the amounts eluted were estimated as agglutinating units.

One ml of packed sensitized erythrocytes corresponding to 50 agglutinating units when tested in tubes, were subjected to elution using the batch procedure. No significant difference in recovery was found. Further, when malignant tissue was eluted, there was no difference between 3 of the heat eluates whether they were centrifuged at 56° C or at

TABLE 1 *Eluted IgG in mg/kg Wet Homogenized Tissue from Different Tumours using Different Procedures*

Elution procedure	Origin of carcinoma				
	colon	uncertain origin	breast	ovary	bronchus
I Batch					
A	9	12	6	7	12
B	9	6	10	6 5	12
C	9	3 5	10	<2.5	7
D	4	4	6	<2.5	5
E	3 5	<2 5	6	<2 5	4
II Column	51	85	55	73	80

A The sediment was resuspended in twice the volume of PBS, incubated at 56° C for 30 min and centrifuged at the same temperature as described previously (11)

B As in procedure A, but centrifuged at ambient temperature

C The sediment was resuspended in twice the volume of glycine HCl buffer, pH 2, and left at ambient temperature for 30 min and centrifuged at the same temperature. The eluate was then dialysed for 18 h against 300 volumes of PBS with two shifts of PBS

D The sediment was resuspended in twice the volume of 3 M NaCl, and further treated as in C.

E The sediment was resuspended in twice the volume of 3 M NaSCN, and further treated as in C.

MATERIALS AND METHODS

Tissues

Specimens from 13 different human solid tumours of which 3 were both primary and secondary, and normal liver, spleen, lung, kidney, stomach and muscle were kindly provided by the Department of Pathology, University of Bergen. Normal organs were collected at autopsies of young persons (aged 20 to 30 years) who had died in traffic accidents and older persons (aged 50 to 60 years) who had died of heart attacks without signs of other diseases. Necrotic tissue was carefully removed and discarded. Tissues not used immediately were stored at −25° C. Histological investigation was routinely performed at the Department of Pathology.

Homogenates of Tissue

The tissue was gently minced with scissors and homogenized for 60 s in phosphate buffered saline, pH 7.2 (PBS) (4 times wet weight) in a Serval Omni-Mixer with the container in an ice-water bath. The homogenate was then centrifuged at 20,000 x *g* for 20 min at + 4° C. The supernatant beneath the lipid layer (extract) was stored at −25° C.

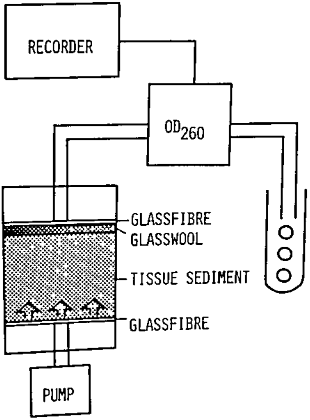


Fig. 1. Illustration of the glass column technique for washing and elution of tissue. V_o = 20 ml, inner diameter = 3.8 cm. Continuous flow from bottom to top with peristaltic pump, flow rate 1.5 ml/min. Optical density (O.D.) recorded at 260 nm.

Eluates of Tissue

1: *Batch procedure.* The homogenized sediment was washed 10 times in 10 vol. of PBS at a centrifugation of 1,000 x *g* for 10 min. The tissue sediment was resuspended in twice the volumes of PBS (w/v) and left at ambient temperature (approx. 20° C) for 30 min. After centrifugation the supernatant was saved for control purposes. The sediment was then divided into portions of 3 g of wet weight and eluates made as stated in Table 1.

The controls and eluates were concentrated 20 times in collodium bags using negative pressure (4, p. 729).

2. *Column procedure.* Three grams of the wet tissue sediment were resuspended in PBS with 0.05 per cent Na-azide and placed in a short glass column using glass fiber filters (Skatron, Norway) at the lower end, and the same filters together with glass wool at the upper end (Fig. 1). In this column the tissue was washed and eluted in a continuous upward flow of PBS with azide. The filter at the bottom issues an even stream and the glass wool at the top prevents the filter from tightening. To monitor the washing and elution procedure the optical density (OD) was recorded at 260 nm and the effluent collected. The tissue was washed at + 4° C until the OD was stable below 0.05; 18 h was the usual time needed. The flow was then stopped for 30 min and a control sample of approximately 60 ml was then collected. Afterwards, the column and 40 cm of the afferent tubing was submerged in a water-bath at 37, 45 and 56° C, successively. Monitored by the OD of the effluent, the eluates were collected at each of the temperatures (see Fig. 2). Before the elution was started, the flow was stopped for 30 min for stabilization at each of the temperatures. All samples were concentrated to between 1.5 and 2.5 ml using Amicon UM 05 filters.

Sera

Sera were obtained from 2 patients with malignant tumours and from 2 of the patients from whom 5 normal organs were collected. The sera were treated with 2-mercaptoethanol (ME) by incubating a mixture of equal volumes of serum diluted 1 in 2 and 0.2 M ME for 30 min at 37° C.

Antiserum to human immunoglobulins was produced as described previously (7). The antiglobulin titre of a serum was defined as the reciprocal value of the highest dilution which agglutinated rabbit erythrocytes sensitized by one half agglutinating unit of human IgG (see below). Antiserum to sheep erythrocytes was raised in rabbits by intravenous immunization. FITC conjugated rabbit anti-human IgG, IgA, IgM, kappa and lambda (lot 047) and FITC conjugated swine anti-rabbit IgG (lot 125) were purchased from Dako-Immunoglobulins, Copenhagen.

Isolated human IgG was purchased from AB Kabi, Stockholm. The preparation contained less than 10 per cent IgA.

Erythrocytes

Erythrocytes from sheep and rabbits were obtained from whole blood collected in Alsever's solution. Before use the erythrocytes were washed 4 times in 6 to 10 volumes of PBS. They were finally centrifuged at 1000 x

TABLE 3. *Amounts of IgG and Ratios of Agglutinins to Concentrations of IgG in Eluates of Human Malignant Tissues*

Origin of tumour	Localization	IgG[a]	Ratio
Carcinoma of			
A uncertain origin	lung	85	–
B the colon	liver	51	R
C the bronchus	primary	130	S
C the bronchus	kidney	160	S
D the bronchus	primary	80	–
D the bronchus	liver	80	–
E the bronchus	primary	60	–
E the bronchus	liver	65	–
F the breast	liver	55	S
G the breast	liver	30	–
H the ovary	peritoneum	73	–
I the cervix	liver	180	R
J the endometrium	liver	27	–
K the bladder	liver	90	–
Fibrosarcoma			
L	primary	110	–
M	primary	10	–

[a] mg/kg wet homogenized tissue
– agglutinins not detected
S ratio in eluates similar to that in extract
R ratio reduced

of tumour A was 1:6. The 56°C eluate had high concentrations of IgG and none of the eluates contained agglutinins.

For tumour B the ratio of agglutinins to IgG was reduced from 1.2 in the extract, to 1:8 in the 56°C eluate. No agglutinins were found in the 37 and 45°C eluate.

The ratio of agglutinins to IgG in tumour C, a carcinoma of the bronchus, was similar in the extract and all the eluates.

Tumour D is another carcinoma of the bronchus with low concentration of IgG in the 56° C eluate and no agglutinins detectable in any of the eluates.

As the volumes of the eluates were not constant, the total amount of IgG eluted was calculated in order to compare the various tissues. Results obtained for all tumours are presented in Table 3. The amount of IgG eluted varied to a large degree, but except for 3 tumours, more IgG was eluted than from normal organs (see below), if the results obtained with kidneys from older individuals were excluded Agglutinins to rabbit erythrocytes were detected in 4 of the 13 tumours of which 2 (C + F) had the same ratio and 2 (B + I) had reduced ratio of agglutinins to IgG, when compared to the extract. The results differed from tumour to tumour, also within the tumours from the same organs of the same histological classification (C + D). On the other hand, the primary and secondary tumours gave similar results (tumours C, D and E).

Eluates of Normal Tissue

A total of 5 kidneys, 3 livers, 3 spleens, 2 lungs, 2 stomachs and 2 quadriceps muscles from 10 different individuals were included. Representative results of the elution experiments are shown in Table 4. As previously (15), all extracts contained IgG and agglutinins to rabbit erythrocytes. By calculation of the ratio of titres of agglutinins to concentration of IgG, the extracts of different organs from the same individual had a constant ratio, but the ratio differed from person to person

The eluates from all livers and spleens tested contained neither IgG nor agglutinins. Eluates from lung, stomach and muscle contained small amounts of IgG, and some of them also agglutinins. There was no difference in these results between young and older individuals. In the eluate from the kidney, neither IgG nor agglutinins were detectable from younger individuals, but both were found in eluates from older individuals. The ratio of titre of agglutinins to concentration of IgG was similar

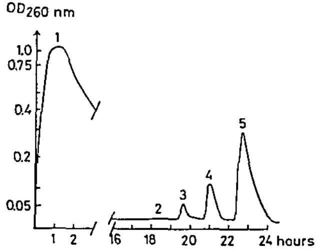


Fig. 2. Diagram from a typical elution experiment using the continuous flow technique.
1: washing, 2: control, 3: elution at 37° C, 4: elution at 45° C, 5: elution at 56° C.

ambient temperature. The best recovery of IgG was obtained using heat elution (A and B, Table 1).

However, as much as 9 times more IgG was eluted using the column procedure (Table 1). The diagram of the OD recorded from a typical elution experiment using this technique is shown in Fig. 2.

After this elution at 56° C, the tissue was in some experiments further eluted using 3 M NaSCN and buffer pH 2 at ambient temperature, PBS at 63° C, or 3 M NaCl at 37° C. Only trace amounts of IgG were found, and this inconsistently.

Two experiments included incubation of the tissue sediments suspended in twice the volume of PBS containing DNase (0 15 mg/ml) and collagenase (0.5 mg/ml) for 2 h at 37° C before washing and elution to exclude non-specific binding to DNA. This treatment did not change the results.

In some experiments, the temperature was measured within the column. After 5 min the temperature was 3° C below the water-bath temperature, and after 20 min it had reached the same temperature. The temperature was constant during the elution.

More than 80 per cent of the known antibodies were recovered at 56° C. Only smaller amounts of the natural antibodies were eluted at 45° C. Examination of the sensitized erythrocytes using FITC conjugated antisera showed a spotted membrane staining of the sensitized erythrocytes, and no change was observed in the staining after elution at 37 or 45° C. No staining was visible on unsensitized cells or sensitized cells treated at 56° C.

To check the reproducibility of the elution procedure, 9 of the tumours were eluted twice or more. One tumour was tested 4 times and the eluates gave a mean value of 51 mg IgG/kg wet tissue homogenate in a range from 40 to 66. Similar results were obtained using the other tumours, resulting in IgG quantities ranging from 10 to 180 mg/kg wet tissue. The tissue was weighed after the elution. Less than 5 per cent of the tissue was lost during the elution procedure.

Agglutinins in Sera and Extracts

Agglutinins to rabbit erythrocytes were detected in the sera, both of IgG and IgM class, as previously (13). The ratio of titre of the IgG antibodies to the concentration of IgG was similar in the serum and the corresponding extracts, both using normal and malignant tissue.

Eluates of Malignant Tissue

Extracts of all the tumours contained detectable amounts of IgG and agglutinins to rabbit erythrocytes as previously described (15). Further, IgG was found in all the eluates, and most IgG was eluted at 56°C. With regard to the agglutinins to rabbit erythrocytes, 4 different patterns appeared, represented by the 4 carcinomas listed in Table 2. The calculated ratio of agglutinins to IgG in the extracts

TABLE 2. *Titres of Agglutinins to Rabbit Erythrocytes and Concentrations of IgG in Extracts and Eluates of 4 Carcinomas*

Origin of carcinoma	Localization	Extract		Eluate 37°C		Eluate 45°C		Eluate 56°C	
		Titre	IgG[a]	Titre	IgG[a]	Titre	IgG	Titre	IgG
A uncertain origin	lung	4	250	<1	30	<1	50	<1	200
B colon	liver	16	350	<1	<12.5	<1	32	2	170
C bronchus	kidney	32	600	1	21	2	28	8	210
D bronchus	liver	8	350	<1	<12.5	<1	51	<1	55

[a] mg/l of extract or eluate.

the IgG associated with these tumours are non-specifically bound as IgG antibodies to rabbit erythrocytes were detected in eluates from 4 tumours In the eluates of 2 of the tumours (C + F, Table 3), there was a similar ratio of agglutinins to IgG (pattern C, Table 2) indicating a predominance of non-specifically bound IgG. In the eluates from 2 other tumours (B + I), there was a reduced ratio (pattern B), indicating both specifically and non-specifically bound IgG. In the remaining 9 tumours, the IgG concentration was too low in 8 for agglutinins to be detected, and in 1 (A) there was a high IgG concentration without agglutinins, indicating a predominance of specifically bound IgG eluted

Most of the non-specifically bound IgG was eluted at 56° C, indicating a rather strong binding. Elution at 37° C and 45° C gave no additional information. We consider it sufficient to perform the elution at 56° C

The eluates of spleen, liver and kidney from younger individuals contained neither IgG nor agglutinins This is consistent with previous findings (11) Eluates of lung, stomach, muscle and kidney from older individuals contained IgG, and agglutinins were present in some of them. Most of the IgG were eluted at 56° C. Where agglutinins were detected, the ratio of agglutinins to IgG was similar in extracts and eluates Evidently IgG is non-specifically bound as expected

It is difficult to say to which of the cells in the tumour the non-related IgG is bound. But since neither IgG nor agglutinins were eluted from normal spleen and liver, and only smaller amounts of IgG were eluted from lung and stomach, it is most likely that the non-related IgG is eluted from the tumour cells and not from the infiltrating host cells However, since lymphoid cells and probably tumour cells possess a Fc-receptor which can bind IgG, further investigations are needed to clarify this point

With regard to the eluates of the kidney from older individuals, it is difficult to explain the results obtained Some of the IgG could be bound to the C3b receptor recently described (2, 8), but obviously other kinds of non-specific binding of IgG must exist.

The results obtained here show a great pleomorphism even for the same type of tumour from different individuals (tumour C + D, Table 2 and 3). The only constant finding is that primary and secondary tumours within the same patient behave similarly This implies that larger secondary tumours can be used for such investigations, with the knowledge that the results obtained are representative for the primary tumour.

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TABLE 4. *Mode Titres of Agglutinins to Rabbit Erythrocytes and Concentrations of IgG in Extracts and Eluates of Normal Organs*

Normal organs	Extract		Eluate 37°C		Eluate 45°C		Eluate 56°C		Total IgG[b]
	Titre	IgG[a]	Titre	IgG[a]	Titre	IgG[a]	Titre	IgG[a]	
A kidney	16	550	<1	<12.5	<1	<12.5	<1	<12.5	<7.5
A liver	8	250	<1	<12.5	<1	<12.5	<1	<12.5	<7.5
A spleen	8	270	<1	<12.5	<1	<12.5	<1	<12.5	<7.5
B kidney	4	110	1	22	2	65	4	110	80
C stomach	8	550	<1	16	<1	21	1	80	30
C lung	4	300	<1	<12.5	<1	14	1	75	25
D muscle	8	170	<1	<12.5	<1	<12.5	1	34	10
E liver	2	250	<1	<12.5	<1	<12.5	<1	<12.5	<7.5

[A] person age 20 years.
[B, C, D, E] persons aged 50–60 years.
[a] mg/l.
[b] mg/kg wet homogenized tissue.

both in extract and in the eluates where the agglutinins were found.

DISCUSSION

We have compared some of the techniques used for elution of immunoglobulins from homogenized tissues. The most extensive elution was obtained at 56° C when the tissue was suspended in a continuous flow of PBS as compared to the batch elution: up to a ninefold increase of quantity of IgG was obtained. No more IgG could subsequently be released from this tissue using any of the other methods. Since elution with PBS at 56° C is a gentle treatment of the tissue, and dialysis is unnecessary, the described procedure was preferred. Further, pretreatment with DNase and collagenase had no influence on the results. The temperature rose quickly inside the column after incubation was started, and remained constant during the elution procedure. Less than 5 per cent of the tissue was lost. Antibodies coated on formalinized erythrocytes were mainly eluted at 56° C. However, a smaller amount of the natural antibodies to rabbit erythrocytes was also eluted at 45° C, perhaps because of lower avidity of these antibodies. Elution of the same type of tissue several times gave an acceptable reproducibility. The elution procedure described should therefore be an improvement compared to other techniques used. However, we are aware of that the batch procedure was not fully investigated. The loss of smaller fragments by washing of the tissue at 1,000 × g and the use of small volumes of eluant, can explain the low recovery of IgG. However, it is difficult to achieve the advantage by monitoring the elution process as obtained with the column procedure.

We have previously reported (15) that agglutinins to rabbit erythrocytes found in extracts of malignant and normal human tissues are identically to the naturally occurring IgG antibodies to rabbit erythrocytes found in serum. These antibodies have no allo- or auto-reactivity (13). One can then either compare the ratio of IgG concentrations in eluates, extracts and serum or in different extracts to the ratio of corresponding titre of agglutinins; or one can compare the ratio of titre of agglutinins to the concentrations of IgG in serum, extracts or eluates, which we have chosen as most convenient.

The ratio of agglutinins (IgG antibodies) to IgG was similar in serum and corresponding extracts. The ratio was constant using extracts from different organs from the same person, but differed from person to person. Since IgM antibodies have not been detected in extracts (15), the extracts were considered representative for the IgG in the extracellular compartment of the tissue. These agglutinins can therefore be used as marker antibodies for non-specifically bound IgG in tissue eluates. By comparing the ratio of agglutinins to IgG in extracts and eluates, the amount of non-specifically bound IgG can be estimated.

IgG was eluted from all the malignant tissues as previously (11). The amount varied to a large degree, but the malignant tissue contained more IgG than most of the normal tissues. Evidently some of

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Fcγ RECEPTORS AND IgG ASSOCIATED WITH HUMAN MALIGNANT TUMOURS

FINN WESENBERG

Broegelmann Research Laboratory for Microbiology, University of Bergen, Bergen, Norway

Wesenberg, F Fcγ receptors and IgG associated with human malignant tumours Acta path microbiol. scand Sect C, *86* 259–263, 1978

Thirteen solid tumours were tested for Fcγ receptors (FcR) using tissue sections and sheep erythrocytes sensitized with rabbit antibodies as indicator cells No alteration was found in FcR activity (FcRA) between untreated tissue and tissue homogenized for 2 min or less More extensive homogenization abolished the FcRA Tissues homogenized for 1 min were washed at 4° C and eluted at 37, 45 and 56° C The FcRA was not altered after elution at 37 and 45° C However, elution at 56° C abolished the activity indicating that the FcR were either eluted or destroyed Tumours which showed a diffuse pattern of FcRA and no non-specifically bound IgG displayed an inverse relationship between the FcRA and the eluted IgG. Such relationship was not found with tumours containing non-specifically bound IgG

Key words human tumours, Fcγ receptor, tumour-associated IgG

F Wesenberg, Broegelmann Research Laboratory for Microbiology, MFH-bygget, N-5016 Haukeland sykehus, Bergen, Norway

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There is a large body of evidence showing the existence of tumour-associated immunoglobulins, but if these immunoglobulins are specifically bound to tumour associated antigens have not always been adequately determined (for review, see refs 9 and 15) However, non-related immune complexes can bind to cells from a non-lymphoid murine tumour (1), and to human acute myeloid leukaemic leucocytes (7) Further, non-specifically bound IgG can be eluted from human solid tumours (14)

The question therefore arose whether the non-specifically bound IgG was attached to the Fcγ receptor (FcR) associated with the malignant tissue. As the FcR-bearing cells in murine solid tumours can be of host origin (5), and the FcR activity (FcRA) can be used as a reliable marker for quantification of infiltrating host cells (4), the non-related IgG can be bound to the host cells (1).

However, there is still a question whether only host infiltrating mononuclear cells in human solid tumours are the FcR-bearing cells or whether the malignant cells also carry the receptor. Nevertheless, human tumour-associated FcR can bind IgG. *Tønder et al.* (12) found an inverse relationship between the amount of IgG on tumour cells in suspension and the FcRA in tissue sections, indicating that the IgG binds with the Fc part to the cells and blocks the binding of the indicator cells.

The present study was designed to investigate whether there was any relationship between the amount of IgG eluted from the tissue and the FcRA in corresponding tissue sections.

MATERIALS AND METHODS

Tissue Specimens from 2 fibrosarcomas and a total of 11 carcinomas from the colon, breast, bronchus, ovary, endometrium, cervix and bladder, of which 3 included both primary and secondary deposits, as well as normal liver and spleen, were the same tissues as used previously (14) Necrotic tissue was carefully removed and discarded, and suitable pieces of the tissue were frozen in liquid nitrogen and mounted on specimen holders for the preparation of tissue sections. The remainder of the tissues were frozen and stored at −25° C.

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TABLE 2 *Adsorption of EA[a] to Sections of Tissue Sediments from a Carcinoma of the Colon Eluted at Differing Temperatures*

Tissue preparations	Agglutinating units of A on EA			
	2	1	½	¼
Untreated	+ + +	+ +	+	–
Hom[b]	+ + +	+ +	+	–
Hom + washed at 4° C	+ + +	+ +	+	–
Hom + eluted at 37° C	+ + +	+ +	+	–
Hom + eluted at 45° C	+ + +	+ +	±	–
Hom + eluted at 56° C	–	–	–	–

[a] sheep erythrocytes (E) sensitized with varying amounts of rabbit antibodies (A)
[b] tissue homogenized for ½ min.

change the activity. Longer homogenization of the tissue partly abolished the activity, and after 8 min no activity remained. Disruption of the tissue in a bacterial press always abolished the FcRA in the tissue. Similar results were obtained with normal and malignant tissues. This implies that for the elution experiment the tissue should be homogenized for less than 2 min in order to preserve the FcRA.

FcRA in eluted tissue. Homogenized malignant tissue was subjected to heat elution and tested for FcRA. Representative results from one such experiment are shown in Table 2. The FcRA did not change significantly between untreated tissue and tissue homogenized, washed or eluted at 37° C or 45° C. However, after treatment at 56° C no FcRA was retained. Similar results were obtained with normal liver and spleen.

Agglutination of rabbit erythrocytes. When sections of untreated tissue were tested for agglutination of rabbit erythrocytes, agglutinates ranging from 3 + to 1 + were observed (13). Most of the tissues gave a 2 + reaction. No agglutinates were observed on sections of homogenized, washed or eluted tissue. Three of the tumours adsorbed erythrocytes to a slight degree. No difference was found whether the tissue was untreated, homogenized, washed or eluted. No erythrocytes were attached after 24 h at 4° C.

Relation between FcRA and eluted IgG. The FcRA in sections from untreated tissue differed between the tumours, as did the pattern of the adsorbed EA: some gave diffuse, other a focal pattern.

When the FcRA in tissue sections from all the tumours was plotted against the total amount of eluted IgG, no definite relationship was found (Fig. 1a). However, the 2 tumours (of which 1 tumour is both primary and secondary, both results plotted) which had predominantly non-specifically bound IgG, adsorbed EA sensitized with $^1/_8$ and $^1/_{16}$ agglutinating unit, despite that 1 of the tumours had 3 times as much IgG in the eluate as the other. The 2 tumours having both non-specifically and specifically bound IgG, adsorbed EA sensitized with ½ and ¼ agglutinating unit, and again 1 of them had 3 times as much IgG in the eluate as the other. The 6 carcinomas (2 of them both primary and secondary, both results plotted) containing no non-specifically bound IgG, but where the IgG concentrations in the eluates were too low for agglutinins to be found, had a tendency to group along the line, indicating an inverse relationship between free FcR and eluted IgG (broken line, Fig. 1b). This tendency was especially marked in the 3 tumours (1 of them both primary and secondary, both results plotted) which had a diffuse pattern of adsorbed EA (unbroken line Fig. 1b). The 2 fibrosarcomas showed a direct relationship between free FcR and eluted IgG. There was no difference in the results obtained using primary tumours or their secondary deposits. Normal liver and spleen had no detectable IgG in the eluates, and these results were therefore not plotted.

Homogenized tissue. Homogenization of the tissue in phosphate buffered saline pH 7.2 (PBS) and preparing of extracts was done as previously (13, 14), using a Servall Omnimixer with the container in an ice-water bath. Disruption of the tissue was also done with an X-press (2). The press containing pieces of tissue was stored at −25° C for more than 12 h followed by 30 min at −70° C before disruption.

Demonstration of FcRA in tissue sections. The procedure with frozen tissue sections was the same as that described by *Tønder et al.* (11) using the closed chamber technique. The indicator cells (EA) were sheep erythrocytes (E) sensitized with rabbit IgG antibodies (A) in varying amounts expressed as agglutinating units. One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of a 1 per cent suspension of E. The indicator cells were suspended to a 1 per cent suspension in PBS containing ½ per cent normal rabbit serum. The concavity of a microculture slide was filled with the EA. A large coverglass with a cryostat section of the tissue was placed over the concavity. The slide was inverted to allow the EA to settle onto the section, and was incubated for 30 min at room temperature. The slide was then turned and left for the detachment of indicator cells from the glass and non-reactive tissue, and the degree of haemadsorption (+ + +, + +, +, −) was recorded microscopically when the glass around the tissue was free of erythrocytes.

The grading of the FcRA was recorded by referring to the highest dilution of A used to prepare EA which gave reaction.

Homogenized, disrupted and eluted tissue was centrifuged for 10 min at 1000 × *g*. The sediment was frozen at −25° C and mounted on specimen holders. Cryostat sections were made as described above.

Agglutination of rabbit erythrocytes. Agglutination of rabbit erythrocytes on tissue sections using the closed chambers or 'open' glass slides, and agglutination of rabbit erythrocytes in tubes and antiglobulin titre were recorded as previously (13, 14).

Tissue eluates. Heat eluates of homogenized tissues from the 13 tumours, spleen and liver, were the same as used previously (14). In brief, the eluates were made by suspending the homogenized tissue in PBS between glass-fiber filters in a short glass column. The tissue suspension was washed at 4° C and eluted at 37, 45 and 56° C successively in a continuous flow of PBS containing 0.05 per cent of Na-azide. After each step, small parts of the tissue were removed from the column and tested for FcRA. The eluates were concentrated and the IgG was quantified using the single radial immunodiffusion technique using commercial agar plates (Behringwerke A.G., Marburg-Lahn, West Germany). The titre of agglutinins to rabbit erythrocytes were recorded, as these agglutinins are identical to the natural occurring serum IgG antibodies (13), and served as the marker-antibodies for non-specifically bound IgG. By comparing the ratio of the titre of agglutinins to the concentration of IgG (mg/l) in eluates and extracts, the degree of non-specifically bound IgG could be estimated. Of the 13 tumours, agglutinins were detected in 4, of which 2 had the same ratio in the eluates as in the extract, indicating a predominance of non-specifically bound IgG and 2 had reduced ratio, indicating the presence of both specifically and non-specifically bound IgG. Of the remaining 9 tumours, 8 contained no detectable non-specifically bound IgG, but the concentration of IgG in the eluates was too low for agglutinins to be detected, and one tumour contained large amounts of IgG but no marker antibodies, indicating a predominance of specifically bound IgG eluted.

RESULTS

FcRA in homogenized tissue. Tissue homogenized for ½, 1, 2, 4, and 8 min was tested for FcRA (Table 1). Homogenization for up to 2 min did not

TABLE 1. *Adsorption of EA[a] to Sections of Tissue Sediments from a Carcinoma of the Colon Using Differing Disruption Procedures*

Tissue preparations	Agglutinating units of A on EA			
	2	1	½	¼
Untreated	+ + +	+ +	+	−
Homogenized for. ½ min	+ + +	+ +	+	−
1 min	+ + +	+ +	+	−
2 min	+ + +	+ +	±	−
4 min	+	±	−	−
8 min	−	−	−	−
»Pressed«[b]	−	−	−	−

a) sheep erythrocytes (E) sensitized with varying amounts of rabbit antibodies (A)
b) tissue disrupted in a bacterial press (X-press).

Where non-specifically bound IgG was found, the tissues had rather high FcRA independent of the IgG eluted This means that 1) the non-specifically bound IgG are not attached to the FcR as indicated above, 2) Shedded FcR (3, 6, 8) could bind to the specifically bound IgG on tumour cells, and if the receptors are polyvalent (6), they could bind non-related IgG, as well as indicator cells This would give a high or low content of both IgG and marker antibodies, depending on the amount of the specifically bound IgG, and would, if free FcR were still available, adsorb EA to a high degree. This agrees with the results presented here. 3) The FcR detected in tissue sections could also be internal FcR, and if they have not bound IgG since they are not exposed to them *in vivo*, there would be no relation between FcRA and eluted IgG.

The FcRA was similar in primary tumours and their secondary deposits, supporting our findings (14) that larger secondary tumours can be used in our experiments

Rabbit erythrocytes were agglutinated on all untreated tissue sections, supporting previous results (13), but none of the homogenized, washed or eluted tissues had this activity. With regard to the 3 tumours which adsorbed rabbit erythrocytes to a slight degree, no relationship was found to the IgG content or to marker antibodies in the eluates, and the eluted tissues behaved similarly to the untreated tissues Hypothetically, non-specifically bound IgG with specificity for rabbit erythrocytes could bind these erythrocytes.

In conclusion, the tumour-associated IgG can be 1) specifically bound to tumour associated antigens (reviewed in 9 and 15), 2) non-specifically bound to FcR in the tissue, and 3) non-specifically bound but not attached to FcR

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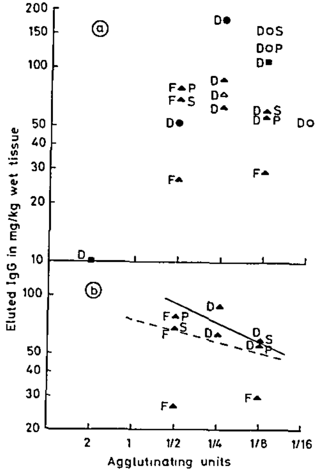


Fig. 1

a) Amount of eluted IgG in mg/kg wet tissue plotted against Fcγ receptor activity in sections of the same tumours from 11 carcinomas and two fibrosarcomas. Diffuse (D) and focal (F) attachment of EA to tissue sections.

○ marker antibodies eluted in the same ratio as in extract, indicating a predominance of non-specifically bound IgG

● marker antibodies eluted in reduced ratio compared to extract, indicating both specifically and non-specifically bound IgG

△: marker antibodies not found, indicating a predominance of specifically bound IgG

▲. marker antibodies not found, but the IgG concentration too low for marker antibodies to be found.

■· fibrosarcomas, but results as for ▲

Three of the tumours were both primary (P) and secondary (S).

b) As a), but only ▲ plotted
Broken line. median values of all the tumours
Unbroken line· median values of the tumours which gave D attachment

DISCUSSION

The results obtained showed that the FcRA within tumours disappeared when the tissue was homogenized for more than 8 min, or when the tissue was disrupted in a bacterial press. To retain the activity in the tissue, careful treatment is necessary. I have chosen extensive mincing with scissors and homogenization for 1–1½ min. The FcRA was then always unchanged in homogenized tissue compared to untreated tissue.

There was no increase in the FcRA in the tissue sections after elution at 37° and 45° C in any of the tumours investigated, although IgG, and in 2 tumours also non-specifically bound IgG, was eluted at 37° and 45° C (14). From 11 of the tumours too little IgG was eluted for the detection of non-specifically bound IgG. After elution of the tissue at 56° C no FcRA was retained. This is consistent with previous findings of *Tönder & Thunold* (10) who found no activity in tissue sections left in PBS at 56° C. However, we found large amounts of IgG in the eluates, and also non-specifically bound IgG in 4 tumours (14). This implies that 1) the FcR associated with the tissue is either eluted with the IgG or destroyed. The former interpretation is most likely, since the FcRA found in supernatants from human lymphocytes in cultures are resistant to 56° C (6). Other possible explanations are that 2) the non-specifically bound IgG is not attached to the FcR, or 3) the indicator system was not sensitive enough to detect small quantitative differences of FcRA.

The FcRA in normal liver and spleen behaved as that in malignant tissue. However, the eluates from liver and spleen contained neither IgG nor agglutinins (14). The FcR-bearing cells in these organs apparently have no IgG bound to them. These results support the interpretations that the non-specifically bound IgG in the malignant tissue are not attached to the FcR on host cells.

Tönder et al. (12) found an inverse relationship between the amount of IgG on tumour cells in suspensions and the FcRA in tissue sections in 7 out of 10 tumours, indicating that the IgG bound to the tissue blocks the binding of the indicator cells. I found an inverse relationship between eluted IgG and FcRA in only 3 out of 13 tumours, but the amount of eluted IgG in these 3 tumours were too low for non-specifically bound IgG to be detected.

Fig. 1a shows that the result obtained using the tumour from which we have indicated that a predominance of specifically bound IgG was eluted, also would be found at the line plotted in Fig. 1b. But since FcR react stronger with Fc in antigen-antibody complexes or aggregated IgG than with monomeric IgG (10, 11), antigen-antibody complexes are preferable to the marker antibodies I have used. This tumour could therefore have a predominance of antigen-antibody complexes attached. But since only 1 tumour shows this pattern, the results are not presented in Fig. 1b.

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DETERMINATION OF CARCINOEMBRYONIC ANTIGEN (CEA): COMPARISON OF TWO DIFFERENT ASSAYS

HARALD ØRJASÆTER

National Institute of Public Health, Department of Immunology, Oslo, Norway

Ørjasæter, H Determination of carcinoembryonic antigen (CEA) comparison of two different assays Acta path microbiol scand Sect. C, *86* 265–275, 1978

A comparison was made of CEA determination by the CEA-RIAKIT and our slightly modified CEA-Roche assays The normal levels (mean + 2 SD) found in 63 blood donors were 2.3 μg/l and 3.3 μg/l, respectively The inter-assay reproducibility was similar in the range below 15 μg/l, with variations from 0 3 μg/l (1 SD, < 5 μg/l) to 1 4 μg/l (1 SD, 10–15 μg/l) Both methods measured CEA concentrations down to 1 μg/l The ability of the two assays to discriminate between positive and negative values in patients with urogenital and gastrointestinal cancer was similar. The response to recurrences of colorectal carcinoma was also similar, and the CEA fluctuation was parallel after surgery The CEA-Roche assay generally showed good correlation between measured and theoretical values at both low and high levels Under our test conditions, the disparity between indirect and direct values was 14 μg/l ± 8 (n = 4) in the range 18–32 μg/l, and this must be taken into consideration in interpreting the CEA-Roche test The CEA-RIAKIT measured too low values as compared to both the CEA-Roche and the theoretical values The discrepancy increased with increasing CEA values, and the ability to distinguish between high CEA concentrations (>15 μg/l) was poor The explanation seems to be that the anti-CEA sera used in this assay show a lower affinity for plasma CEA than for tumour-extracted CEA Immunochemical differences between sample CEA and CEA used for calibration of the standard curves must be considered in evaluation of CEA assays

Key words Carcinoembryonic Antigen-Assays

H Ørjasæter Statens Institutt for Folkehelse, Postuttak, Oslo 1, Norway

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In our previous studies of plasma CEA we used a slightly modified CEA-Roche assay (8, 9, 27, 28). However this is time-consuming and the procedure is slow due to a long period of dialysis Although dialysis can be avoided and the turn-around time can be reduced by introduction of a special filtration technique this does not reduce the laboratory work significantly (27) The disparity between values obtained by the indirect and the direct CEA-Roche assay further complicates the use of this assay (10, 11, 14, 19)

Introduction of a new CEA assay, is therefore of great interest However, further evaluation and comparison with well known assays are needed, before a new assay can be adapted to an established routine. Comparable results are important in serial determinations for early detection of recurrences (16, 26) which may initiate second-look operations (13, 15, 22), as well as for monitoring radio- or chemotherapy (6, 23).

In this study, our modified CEA-Roche assay and the new CEA-RIAKIT (Abbott) were evaluated further and compared Comparison was made of the normal reference level, the reproducibility and cancer discriminatory ability, as well as the early response to recurrences, and the correlation between measured and theoretical values was studied. The study indicates that immunochemical differences between sample CEA and CEA used in the calibration of standard curves must be taken into consideration in evaluation of CEA tests.

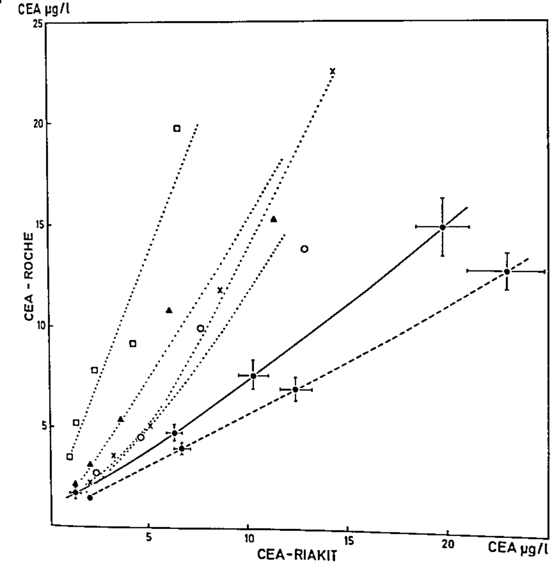


TABLE 1 *CEA Determinations in Reference Samples (R) and in Dilutions of Tumour Extract (K)*

	CEA-Roche	CEA-RIAKIT	Reference or calculated value
R 1	1.7 ± 0.3 (n = 30)	1.3 ± 0.3 (n = 10)	0.7 ± 0.5
R 2	4.8 ± 0.4 (n = 31)	6.4 ± 0.4 (n = 10)	4.5 ± 0.5
R 3	7.7 ± 0.8 (n = 30)	10.4 ± 0.8 (n = 10)	8.0 ± 0.8
R 4	15.0 ± 1.4 (n = 30)	19.8 ± 1.3 (n = 10)	15.0 ± 1.5
K 1	1.2 –	2.2 –	1.7
K 2	4.0 ± 0.4 (n = 10)	6.8 ± 0.4 (n = 10)	3.5
K 3	7.0 ± 0.6 (n = 10)	12.5 ± 0.8 (n = 10)	7.0
K 4	12.9 ± 0.9 (n = 9)	22.9 ± 1.9 (n = 10)	14.0
K 4	[a]33.2 ± 7.9 (n = 5)	22.9 ± 1.9 (n = 10)	14.0
K 5	[a]53.4 ± 3.3 (n = 7)	38.7 ± 2.8 (n = 10)	28.0
K 6	[a]96.6 ± 4.6 (n = 7)	57.8 ± 2.7 (n = 10)	56.0

[a] Direct CEA-Roche assay, the others were performed by the indirect assay

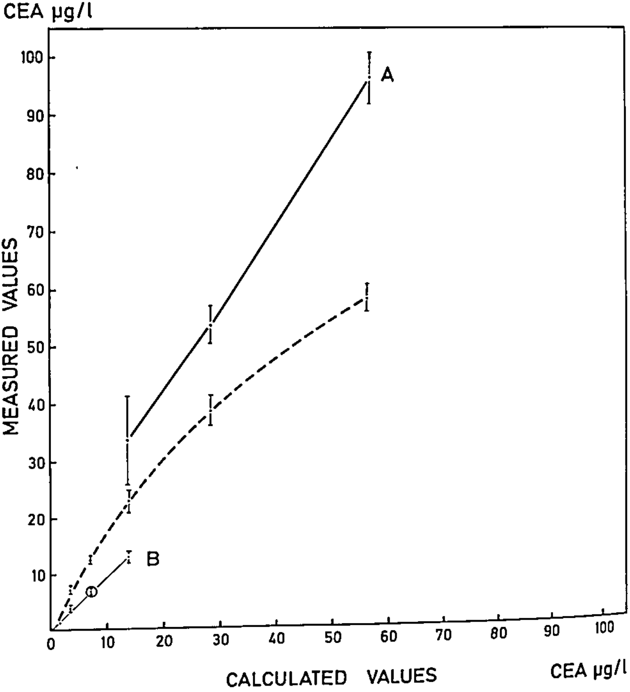


Fig. 1. Comparison of measured and calculated (theoretical) values of CEA in dilutions of PCA extract of liver metastasis from a colonic adenocarcinoma. Twofold dilutions were made in human AB plasma with low CEA level
O Dilution on which the calculation of CEA values is based (mean = 7 µg/l, n = 10)
——A: CEA-Roche assay, direct method.
——B CEA-Roche assay, indirect method
– – – CEA-RIAKIT assay

Fig. 2. Correlation between the indirect CEA-Roche and CEA-RIAKIT measurements
—— Reference samples (Roche).
– – – Dilutions of PCA extract of liver metastasis from a colonic adenocarcinoma
. . . . Four different plasma samples from patients with adenocarcinoma tested in different dilutions

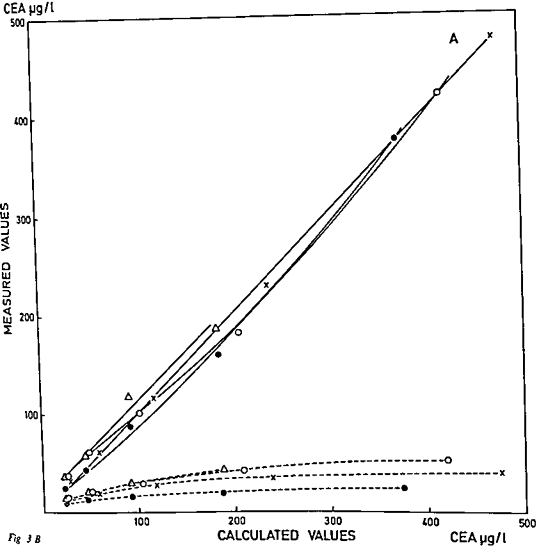


Fig 3 B

Eight patients were monitored by means of serial CEA determinations to detect early recurrence, response to therapy or other fluctuations in the CEA level The patients were selected because of variations in CEA level detected by our CEA-Roche assay The CEA-RIAKIT tests were performed later on the same samples Six of the patients had recurrence of colorectal carcinoma, confirmed by second-look operation in five cases and by clinical examinations in one One patient had liver cirrhosis with exceptionally high CEA level but without any evidence of malignancy and one patient with high CEA level had been under observation for malignant disease for 2 years

The indirect CEA-Roche test was performed as recommended, except for minor modifications described elsewhere (9, 27, 28) Both CEA-standard tubes and the samples were subjected to identical procedures, including PCA extraction The standard solution was prepared from PCA extract of liver metastasis from a colonic carcinoma diluted in normal human plasma 125J CEA and anti-CEA reagents were from the CEA-Roche kit Separation of CEA and anti-CEA complexes was performed by the Z-gel method of *Hansen et al* (5) The direct CEA-Roche test (without PCA extraction) was used to measure CEA concentrations higher than 20 µg/l

Twelve CEA-RIAKITs were used (Nos 109 to 115) The assay was performed according to the instruction for the one-step sandwich method PCA extraction was not used, but the samples were diluted in 0 2 M acetate buffer, pH 5 0, and heat-treated to remove substances which might influence the CEA determination. The heat-treated plasma was incubated with paperdisc coupled anti-CEA and free 125J anti-CEA. Separation of the labelled complexes fixed to the paperdisc was made by a

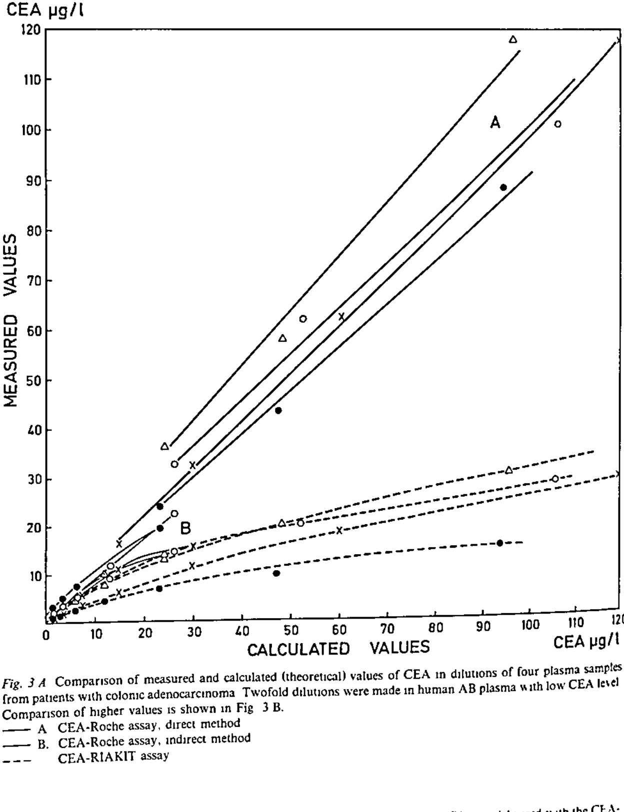


Fig. 3 A Comparison of measured and calculated (theoretical) values of CEA in dilutions of four plasma samples from patients with colonic adenocarcinoma. Twofold dilutions were made in human AB plasma with low CEA level. Comparison of higher values is shown in Fig. 3 B.
—— A. CEA-Roche assay, direct method
—— B. CEA-Roche assay, indirect method
– – – CEA-RIAKIT assay

MATERIAL AND METHODS

This study was performed from April to November 1977. The normal reference level was determined in plasma samples from 63 apparently healthy blood donors. The ability to quantify CEA was studied in four plasma samples with high CEA levels collected from patients with colorectal carcinoma, four reference samples (R) and one sample of perchloric acid (PCA) extract of liver metastasis from a colonic carcinoma (K).

The reference samples (R) were delivered with the CEA-Roche kit (control samples No. 2040 A, 2140 A, 2240 A and 2340 A), these consist of plasma CEA mixed with CEA from extract of liver metastasis from colorectal carcinoma.

The values obtained by the two assays were compared in 115 patients. Seventy-six had gastrointestinal malignancies – the majority colorectal carcinoma – and 39 had urogenital tumours, most of which were carcinoma of the uterine cervix.

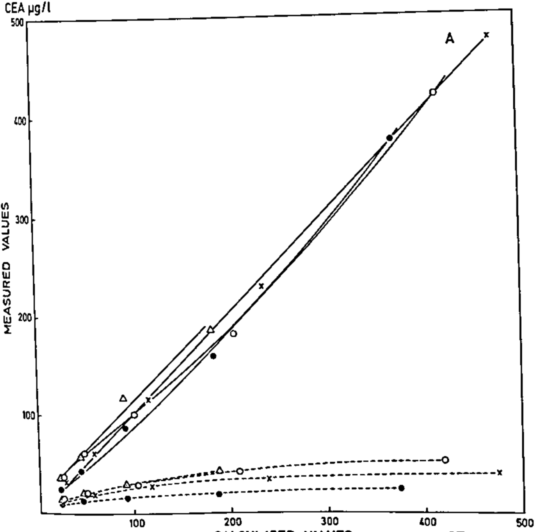


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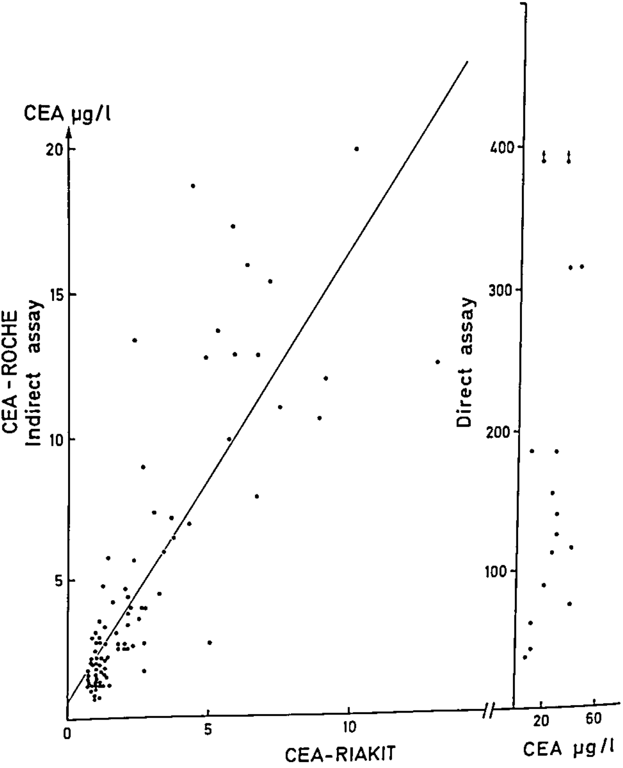


Fig. 4. Correlation between the CEA-Roche and CEA-RIAKIT measurements in 115 plasma samples (Not corrected for difference in the normal reference values)
For CEA $<$ 20 µg/l (CEA-Roche assay)·
n = 99
r = 0.83
y = 1.6x + 0.5

TABLE 2. *Discrimination between Positive and Negative Results in Urogenital and Gastrointestinal Carcinoma*

CEA-Roche / CEA-RIAKIT	Uro-genital carcinoma	Gastro-intestinal carcinoma	Total
Results	No of patients	No of patients	
+ / +	7	37	44
−/−	31	31	62
+ /−	1	6	7
−/ +	0	2	2
	39	76	115

washing procedure Thus, the two methods are based on different principles, and different procedures are used to separate the immune complexes

The tumour tissue extract was diluted with human AB plasma of low CEA level (< 1 µg/l) before being tested, and all serial dilutions of tissue extracts as well as plasma samples were made in this AB plasma Calculation of the theoretical values of CEA was based on determinations by our CEA-Roche assay All samples were stored at − 25° C

The normal reference levels were calculated as means + 2 standard deviations (SD) Reproducibility was given in µg/l corresponding to mean ± 1 SD The linear regression line and Bravais-Pearson correlation coefficient (r) were adopted to show the degree of relationship between CEA-Roche and CEA-RIAKIT values in the low range of measurement (< 20 µg/l)

RESULTS

The mean CEA value in plasma of the 63 blood donors was 1 7 µg/l ± 0 80 measured by our *modified* CEA-Roche assay and 1 2 µg/l ± 0 55 by the CEA-RIAKIT The normal reference levels were 3 3 and 2 3 µg/l respectively Inter-assay determinations of reference samples (R) and tumour extract dilutions (K) are shown in Table 1 and Fig 1 The indirect CEA-Roche values correspond well with both reference and calculated values, while too high values were found with both CEA-RIAKIT and the direct CEA-Roche assays The reproducibility of the two assays was similar in the low range (< 15 µg/l) of measurements where the two assays could be compared

In contrast to the high CEA values measured with the CEA-RIAKIT in tumour extract were the relatively low values found in plasma samples (Fig. 2) In spite of some variation in the four plasma samples, they all had low levels compared with the results found by the indirect CEA-Roche assay The reference samples, which contain plasma CEA as well as CEA from tumour tissues had values lying between those of the tumour extract and plasma samples

Determinations with the CEA-Roche assay correlate well with the calculated values over a wide range of measurements (Fig. 3), but too low values were found by the CEA-RIAKIT in these samples All samples showed disparities between the indirect and direct CEA-Roche values A mean of 18 µg/l ± 4 found by the indirect method corresponded to 32 µg/l ± 5.5 by the direct method. This is due to elevation in the low range of the direct assay, as well as to a tendency for too low values to appear in the upper range of the indirect assay.

The CEA-RIAKIT values correlate fairly well with those of the indirect CEA-Roche assay (r = 0.80 for CEA < 20 µg/l), but different ability to distinguish between increasing CEA levels strongly influenced the correlation at high levels (Fig. 4)

The ability of the two assays to discriminate between positive and negative values in patients with gastrointestinal and urogenital carcinomas was similar, though there was a tendency (not significant) for more positive values to be found by the CEA-Roche assay (*Table 2*)

In Fig. 5 the response of the two assays to early recurrence and to surgical treatment of colorectal carcinoma is compared In all cases the response was similar Also the two patients without known malignant disease had elevated values in both assays

The CEA-RIAKIT did not distinguish well between high CEA concentrations. To overcome this problem, samples were tested in serial dilutions. The estimated CEA value (i e value in diluted sample x dilution factor) was found to increase with increasing dilution until a plateau was reached (Fig

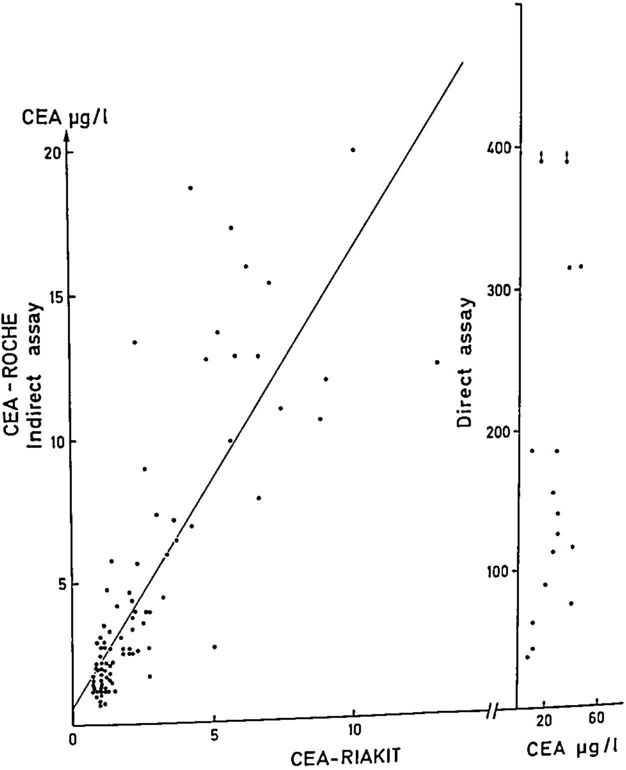


Fig. 4 Correlation between the CEA-Roche and CEA-RIAKIT measurements in 115 plasma samples (Not corrected for difference in the normal reference values)
For CEA < 20 µg/l (CEA-Roche assay)
n = 99
r = 0 83
y = 1 6x + 0 5

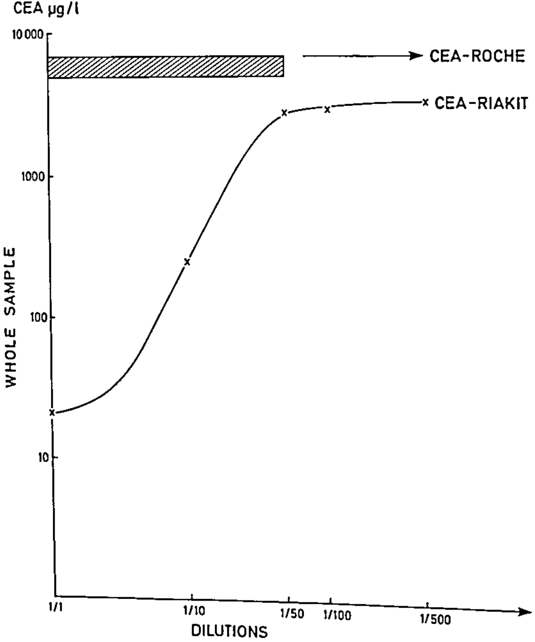


Fig 6 Relation between the dilutions used in the test and the CEA value estimated in the whole sample (value in diluted sample × dilution factor)
——→ CEA-Roche value
▭ Decreased CEA-RIAKIT values

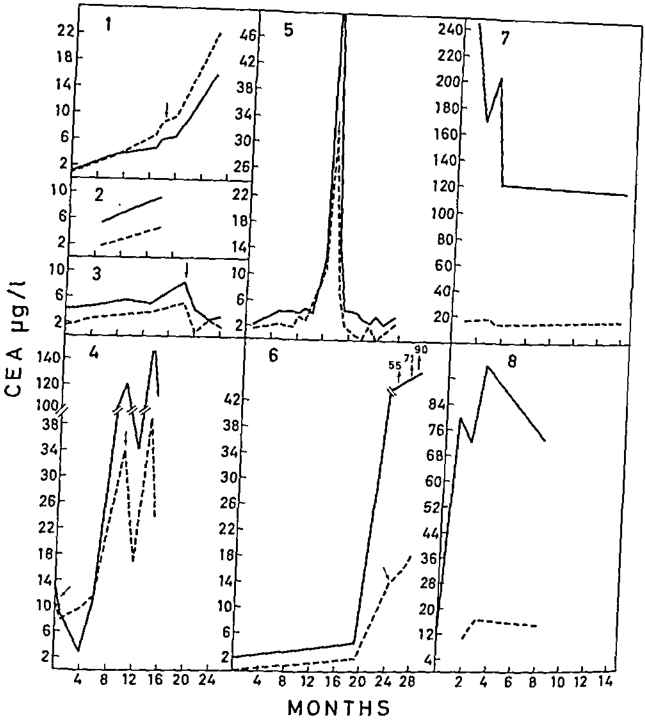


Fig 5. Serial CEA determinations in 8 patients
No. 1–6 Patients with early recurrence of colorectal carcinoma
» 7 Patient with liver cirrhosis (exceptionally high CEA).
» 8 Patient under observation for malignancy
—— CEA-Roche assay.
- - - CEA-RIAKIT assay
| Operation performed

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6). A sample which measured 20 µg/l undiluted was estimated at about 3000 µg/l when tested in dilutions between 1/50–1/500. This compares to a CEA-Roche value of about 7000 µg/l. All the readings in these experiments were based on the accepted part of the standard curve.

DISCUSSION

Under our test conditions, the disparity between indirect and direct CEA-Roche plasma values was 14 µg/l ± 8 (n = 4) in the range between 18–32 µg/l. This is less than those stated in most other reports (10, 11, 14,19). In other studies we have noticed higher disparities in some samples from cancer patients, as would be expected if high level of glycoproteins or cross-reacting materials present in the non-extracted sample had influenced the reaction (12, 28). It may also be due to differences in preparation of CEA-standards and adjustment of standard curves. The interpretation of exact plasma values will be impossible between 20–50 µg/l, but such high CEA values are in any event indicative of a large tumour mass or metastases (13), and an exact CEA value is of less importance. In the other ranges of measurements, our CEA-Roche assay shows good correlations with the theoretical values, which will allow monitoring of radio- or chemotherapy in patients with high CEA levels.

Our CEA-Roche assay has proved valuable for early detection of recurrences, as well as for detection of rest tumours after surgery (13). The CEA-RIAKIT was similar in this respect, but the fluctuation was less marked, and a significant increase may be more difficult to detect with this assay. The CEA-RIAKIT did not distinguish well between high concentrations of CEA, and therefore seems to be less suitable for monitoring patients on radio- or chemotherapy

The normal reference level was slightly lower with the CEA-RIAKIT as compared to the CEA-Roche value, but the ability to discriminate between positive and negative values in urogenital and gastrointestinal cancer was similar. CEA-RIAKIT values of 12 µg/l correspond to 20 µg/l by the CEA-Roche assay. Below these limits the correlation was fairly good ($r = 0.8$), although some adjustment would be necessary if the two results are to be compared The findings are in good accordance with the data reported by *Hirai* (7), who used a similar type of assay. At higher levels the results cannot be compared accurately This is due mainly to the varying ability of the two assays to distinguish between high CEA concentrations.

The standard curve of the CEA-RIAKIT should give good measurements up to 100 µg/l. In spite of this, the level measured in plasma samples never exceeded 40 µg/l, even in samples with CEA concentrations of 3000 µg/l or more. This was in contrast to the high values measured in tumour extract. It is unlikely that the sandwich method as used in the CEA-RIAKIT should be responsible as such for the low plasma values, without effecting the results in tissue extract or standard curve. The different principle used for separation of radiolabelled complexes would also affect equally both standard curves and measurements in tissue extracts. The effect of the heating procedure was not studied and we cannot exclude some difference as compared to the PCA extraction. However, the same heating procedure was used with tissue extract after dilution with human plasma, and thus the selective decrease of plasma CEA values cannot be explained.

The most likely explanation would be that the antibody system in the CEA-RIAKIT assay has low affinity for plasma CEA as compared to tumour-extracted CEA Immunochemical variation and heterogeneity are well known in CEA preparations (4, 12), and differences between plasma and tumour-extracted CEA have been observed in a study with radioimmuno-assay (25) Such differences must be considered when test systems for CEA determination are evaluated, and they seem to be of special interest in assays which use radiolabelled antibodies. In most CEA assays radiolabelled CEA preparations have been used and little attention has been focused on immunochemical differences between plasma and tissue-extracted CEA (1, 2, 3, 7, 17, 18, 20, 21, 24). In all plasma assays, it is important that CEA used for calibration of the standard curve has a reactivity which is similar to plasma CEA.

Further improvement of this assay could be expected if anti-CEA antibodies with a strong affinity for plasma CEA could be introduced, or if a standard curve could be calibrated with plasma CEA. Another possibility is to test diluted samples In samples with high CEA levels, at least two different dilutions must be tested to establish the plateau which gives the right CEA values

We thank *Inger-Lill Svendsen, Randi Grønnvold* and *Kjersti Holte* for skilful technical assistance, and we are indebted to *Sverre Tinnen* and *Svein A Skartsaterhagen* for help with data processing and to *Abbott, Diagnostic Division,* for providing the CEA-RIAKITS

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T-LYMPHOCYTES TRANSFER STREPTOZOTOCIN INDUCED DIABETES MELLITUS IN MICE

KARSTEN BUSCHARD and JØRGEN RYGAARD

Pathological-anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

Buschard, K & Rygaard, J T-lymphocytes transfer streptozotocin induced diabetes mellitus in mice. Acta path microbiol scand Sect. C, 86 277–282, 1978

Diabetes mellitus induced by low dose treatment with streptozotocin in BALB/c mice was passively transferred to syngeneic recipients with a predominantly T-lymphocyte fraction of spleen cells. This fraction was prepared by density centrifugation and passage through an antimouse immunoglobulin coated column Variation in numbers of injected cells in the range of 10^3 to 10^7 showed that the smaller cell numbers gave the highest blood sugar values Athymic nude recipients of BALB/c background also developed diabetes following similar transplants A preliminary result was that the highest blood sugars were seen after transplantation of T-lymphocytes treated by low dose irradiation (200 R) This may reflect a specific effect on T-suppressor lymphocytes

Key words T-lymphocytes, diabetes mellitus, passive transfer; suppressor T cells, nude mice, streptozotocin

Karsten Buschard, Patologisk-anatomisk Institut, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

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This article describes passive transfer of streptozotocin induced diabetes mellitus effected by transplants comprised very largely of thymus derived lymphocytes (T-lymphocytes) In an earlier study (*Buschard & Rygaard* 1977), it was found that chemically induced diabetes mellitus could be passively transferred by transplant of spleen cells, and following pretreatment of the gross spleen extract with antitheta and complement it appeared that the role of the T-lymphocytes was important.

In addition to the passive transfer study as such, we varied the number of T-lymphocytes in the transplant in an attempt to find an optimal for the actual experimental model

Finally, we made transplants of irradiated T-lymphocytes, and of T-lymphocytes to nude mice to derive information, if possible, about the pathophysiological mechanisms

MATERIAL AND METHODS

Donors Ninety four normal male BALB/c/BOM mice, 7–8 weeks old Fifty two received streptozotocin, while 42 functioned as buffer injected controls

Recipients Eighty normal male BALB/c/BOM mice, and 14 nude mice (11th backcross of a gene transfer to BALB/c/BOM), 6–8 weeks old All the animals were purchased from Gl Bomholtgaard, Laboratory Animals Breeding and Research Center, DK-8680 Ry, Denmark. They were reared and kept under specified pathogen free (spf) conditions. During the actual experiments the animals were housed in pairs in Makrolon® type III cages at the Patologisk-anatomisk Institut, Kommunehospitalet, Copenhagen Autoclaved feed pellets (Gl Bomholtgaard), and sterile drinking water were supplied ad libitum

The allocation of animals to the different experimental groups is shown in the tables.

Streptozotocin Treatment and Control

Upjohn (Kalamazoo, Mi, USA) kindly supplied the streptozotocin Donors were injected intraperitoneally with 40 mg/kg BW streptozotocin in citrate buffer (pH 4 2) daily for five days – a total dose of 200 mg/kg BW (*Like & Rossini* 1976) Blood sugar on day 9 was 189 ± 10 ($\pm$ 2SEM) which is somewhat lower than that found previously after administration from other batches of streptozotocin (*Buschard & Rygaard* 1977, *Buschard & Rygaard* 1978) Blood sugar in the buffer injected controls was 98 ± 7. The animals were sacrificed on day 12

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T-LYMPHOCYTES TRANSFER STREPTOZOTOCIN INDUCED DIABETES MELLITUS IN MICE

KARSTEN BUSCHARD and JØRGEN RYGAARD

Pathological-anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

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Diabetes mellitus induced by low dose treatment with streptozotocin in BALB/c mice was passively transferred to syngeneic recipients with a predominantly T-lymphocyte fraction of spleen cells. This fraction was prepared by density centrifugation and passage through an antimouse immunoglobulin coated column. Variation in numbers of injected cells in the range of 10^3 to 10^7 showed that the smaller cell numbers gave the highest blood sugar values. Athymic nude recipients of BALB/c background also developed diabetes following similar transplants. A preliminary result was that the highest blood sugars were seen after transplantation of T-lymphocytes treated by low dose irradiation (200 R). This may reflect a specific effect on T-suppressor lymphocytes.

Key words: T-lymphocytes, diabetes mellitus, passive transfer, suppressor T cells, nude mice, streptozotocin

Karsten Buschard, Patologisk-anatomisk Institut, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

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This article describes passive transfer of streptozotocin induced diabetes mellitus effected by transplants comprised very largely of thymus derived lymphocytes (T-lymphocytes). In an earlier study (*Buschard & Rygaard* 1977), it was found that chemically induced diabetes mellitus could be passively transferred by transplant of spleen cells, and following pretreatment of the gross spleen extract with antitheta and complement it appeared that the role of the T-lymphocytes was important.

In addition to the passive transfer study as such, we varied the number of T-lymphocytes in the transplant in an attempt to find an optimal for the actual experimental model.

Finally, we made transplants of irradiated T-lymphocytes, and of T-lymphocytes to nude mice to derive information, if possible, about the pathophysiological mechanisms.

MATERIAL AND METHODS

Donors. Ninety four normal male BALB/c/BOM mice, 7–8 weeks old. Fifty two received streptozotocin, while 42 functioned as buffer injected controls.

Recipients. Eighty normal male BALB/c/BOM mice, and 14 nude mice (11th backcross of a gene transfer to BALB/c/BOM), 6–8 weeks old. All the animals were purchased from Gl Bomholtgaard, Laboratory Animals Breeding and Research Center, DK-8680 Ry, Denmark. They were reared and kept under specified pathogen free (spf) conditions. During the actual experiments the animals were housed in pairs in Makrolon® type III cages at the Patologisk-anatomisk Institut, Kommunehospitalet, Copenhagen. Autoclaved feed pellets (Gl Bomholtgaard), and sterile drinking water were supplied ad libitum.

The allocation of animals to the different experimental groups is shown in the tables.

Streptozotocin Treatment and Control

Upjohn (Kalamazoo, Mi, USA) kindly supplied the streptozotocin. Donors were injected intraperitoneally with 40 mg/kg BW streptozotocin in citrate buffer (pH 4.2) daily for five days – a total dose of 200 mg/kg BW (*Like & Rossini* 1976). Blood sugar on day 9 was 189 ± 10 (± 2SEM) which is somewhat lower than that found previously after administration from other batches of streptozotocin (*Buschard & Rygaard* 1977, *Buschard & Rygaard* 1978). Blood sugar in the buffer injected controls was 98 ± 7. The animals were sacrificed on day 12.

T-lymphocyte Preparation

Immediately after sacrifice of the donors, the spleens were removed and fine dealt in BSS-buffer with surgical tweezers. Connective tissue elements were removed, and the remaining cell material was agitated with a pipette to effect cell separation. The larger particles were sedimented on ice for five minutes. The lymphocytes of the supernatant were isolated by centrifuging for 30 min at 400 G on a Hypaque-Ficoll gradient (Lymphoprep®, Nyegaard & Co A/S, Oslo, Norway) a.m *Böyum* (1968). Lymphocytes were washed once in buffer, incubated with 0.83% NH_4Cl in BSS for 8 min at 5° C and washed three times. The resultant was adjusted to give a lymphocyte concentration of 2×10^7/ml in BSS with 10% fetal calf serum added.

Fractionation of the lymphocytes was affected by passage through antimouse immunoglobulin coated columns (*Rubin & Wigzell* 1973). Dr. *Bent Rubin*, Statens Seruminstitut, kindly provided the columns, which were made as follows: Glass beads of 0.2 mm diameter were coated with a 0.5% solution of mouse immunoglobulin (MIg) in PBS.

The beads were incubated with the MIg at 45° C for 60 min, and at 4° C overnight. Thereafter they were carefully introduced into the glass columns (1.5 × 30 cm Pharmacia®) Excess MIg solution was removed, and the beads washed with twice their volume of PBS. The column was then filled with rabbit anti-MIg in dilution 1:5. The drain was closed and the column incubated for two hours at 4° C Thereafter, the antiserum was washed off, and the suspension of mouse spleen cells (2×10^7 cells/ml) was passed slowly through the column (3 ml/min approx)

After separation in the column, the resultant T-lymphocytes were washed three times in BSS, and the cell numbers were counted. Cell recovery after column passage was up to 44%. The concentration in the different suspensions was adjusted so that the planned number of cells could be given in 0 5 ml suspensions, which were injected intraperitoneally into the recipient mice. The actual numbers of cells given to animals in the different groups are shown in the tables.

Irradiation

10^7 T-lymphocytes from both experimental and control animals were suspended in BSS in concentration of 2×10^6 cells/ml The irradiation was given by an X-ray therapy unit, exposure 200 R at 200 kV, 1.05 mm Cu half value layer. After this treatment viability counting (trypan blue) showed no dead cells.

Blood Sampling and Testing

Blood for glucose determinations was collected in 25 μl pipettes from the paraorbital venous plexus of nonfasting animals. Blood glucose levels were determined enzymatically using glucose oxidase (*Cooper* 1973)

Statistical Studies

The Mann-Whitney U-test was applied to determine significance. The confidence limits are shown as ± 2 SEM.

RESULTS

Results are shown in detail in the tables and shortly described in the following:

Table 1. *Blood Glucose Values in Normal BALB/c Recipient Mice after Transfer of T-Lymphocytes*

No. of cells in the trans-plants	S/B	Days after transfer							
		3 days	6 days	9 days	13 days	20 days	39 days	52 days	66 days
10^3	S	(8) 146 ± 34	(8) 160 ± 38	(8) 185 ± 37	(7) 212 ± 45	(7) 230 ± 41	(7) 226 ± 25	(7) 181 ± 24	(7) 175 ± 1[illegible]
	B	(5) 79 ± 13	(5) 90 ± 25	(5) 90 ± 22	(5) 103 ± 25	(5) 108 ± 27	(5) 112 ± 36	(5) 103 ± 13	(5) 103 ± 1[illegible]
10^4	S	(8) 160 ± 43	(8) 151 ± 46	(8) 177 ± 47	(8) 213 ± 58	(8) 221 ± 60	(8) 267 ± 40	(8) 230 ± 27	(8) 202 ± 17
	B	(5) 112 ± 12	(4) 109 ± 29	(4) 104 ± 22	(4) 101 ± 15	(4) 96 ± 12	(4) 101 ± 13	(4) 90 ± 4	(4) 99 ± 12
10^5	S	(8) 133 ± 21	(8) 146 ± 19	(8) 144 ± 20	(8) 155 ± 18	(8) 170 ± 27	(8) 213 ± 48	(8) 161 ± 24	(8) 151 ± 17
	B	(5) 95 ± 17	(4) 86 ± 15	(4) 92 ± 16	(4) 96 ± 13	(4) 100 ± 16	(4) 113 ± 13	(4) 96 ± 5	(4) 101 ± 14
10^6	S	(8) 121 ± 15	(8) 129 ± 13	(8) 136 ± 21	(8) 160 ± 27	(8) 180 ± 39	(8) 198 ± 36	(8) 174 ± 26	(8) 175 ± 22
	B	(6) 102 ± 13	(5) 110 ± 31	(4) 107 ± 21	(4) 107 ± 18	(3) 105 ± 39	(2) 126	(2) 123	(1) 126
10^7	S	(8) 132 ± 22	(8) 143 ± 24	(8) 151 ± 36	(8) 176 ± 47	(8) 183 ± 53	(8) 180 ± 51	(8) 175 ± 47	(8) 175 ± 39
	B	(6) 93 ± 18	(5) 91 ± 17	(5) 94 ± 13	(5) 100 ± 7	(5) 117 ± 26	(4) 109 ± 25	(4) 110 ± 14	(4) 110 ± 15

S: Streptozotocin treatment of donor mice.

B Buffer treatment of donor mice.

Average blood glucose values of mice in mg/100 ml ± 2 SEM Number in brackets indicate number of mice in each group. Blood glucose values for day 0. 93 ± 9 mg/100 ml

Table 2. *Blood Glucose Values in Nude BALB/c Recipient Mice after Transfer of T-Lymphocytes*

o. of ells i the ans-lants	S/B	Days after transfer							
		3 days	6 days	9 days	13 days	20 days	39 days	52 days	66 days
0^5	S	(8) 136 ± 31	(8) 134 ± 29	(8) 150 ± 37	(8) 188 ± 43	(7) 220 ± 48	(7) 191 ± 34	(5) 165 ± 14	(5) 146 ± 9
	B	(6) 68 ± 7	(6) 79 ± 11	(6) 80 ± 10	(6) 88 ± 8	(6) 99 ± 12	(5) 115 ± 32	(5) 113 ± 19	(5) 112 ± 18

S Streptozotocin treatment of donor mice
B Buffer treatment of donor mice.
Average blood glucose values of mice in mg/100 ml ± 2 SEM. Number in brackets indicate number of mice in each group

Transfer to Normal Mice

As recorded in Table 1, five groups of mice were injected intraperitoneally with, respectively, 10^3, 10^4, 10^5, 10^6 and 10^7 T-lymphocytes from streptozotocin treated donors, while comparable control groups received T-lymphocytes from buffer injected donors. As early as the third day blood glucose in all the experimental groups were raised by comparison with the control groups Most markedly elevated were blood glucose in the groups which had received the smaller numbers of cells (in the 10^3 group $p < 0.01$) Thereafter there was slow increase in blood glucose up to about the ninth day at which time there was a further rise in blood glucose. The maximum values were measured at the 39th day, except for the 10^3 and 10^7 group, where maximum values were measured earlier – on the 20th day. All experimental groups except the 10^6-group displayed significantly higher blood sugar values in at least 7 out of 8 measurements than did controls ($p < 0.01$ in at least 4 measurements of each group). Control mice of the 10^6-group died early, not allowing full significance testing, but p was < 0.05 on both day 6 and 13.

It can be seen that the groups receiving fewer cells (10^3 and 10^4) displayed consistently higher blood sugar values than the other groups, and maximum values in the 10^4 group are significantly higher than maximum values in the 10^6 and 10^7 groups ($p < 0.05$). Blood sugars remain high after two months – highest in the 10^4 group

Transfer to Nude Mice

Table 2 records the effects of T-lymphocyte transfer from BALB/c mice to corresponding nude mice. Blood sugar values follow those in the corresponding group (10^5) of normal mice.

There is again demonstrable elevation of blood sugars on the third day, and relatively slow increase to a clearly diabetic maximum on the 20th day. Experimental animals displayed consistently significantly higher blood sugars than the controls ($p < 0.01$, days 3–20 and $p < 0.02$, days 39–66.

Table 3 *Blood Glucose Values in Normal BALB/c Recipient Mice after Transfer of X-Ray Treated T-Lymphocytes*

No of cells in the t---	S/B	Days after transfer							
		3 days	6 days	9 days	13 days	20 days	39 days	52 days	66 days
	S	(8) 197 ± 34	(8) 192 ± 24	(8) 227 ± 23	(8) 285 ± 26	(8) 310 ± 16	(8) 263 ± 24	(8) 228 ± 24	(8) 216 ± 27
	B	(5) 108 ± 12	(5) 116 ± 19	(5) 127 ± 32	(5) 112 ± 20	(5) 112 ± 15	(5) 108 ± 8	(5) 115 ± 10	(5) 115 ± 14

S Streptozotocin treatment of donor mice
B Buffer treatment of donor mice
Average blood glucose values of mice in mg/100 ml ± 2 SEM Number in brackets indicate number of mice in each group Blood glucose values for day 0 93 ± 9 mg/100 ml.

Role of X-ray Treatment of Donor Cells

Table 3 records the results of the preliminary study, where mice were transplanted with irradiated T-lymphocytes. Blood sugars were relatively high on the third day, and attained a maximum after 20 days.

On all days when measurements were made, there was significant difference between experimental and control mice ($p < 0.01$). Apart from the values on the 39th and 52nd days, blood sugars in the experimental group were consistently higher than in any of the other experiments. When the experimental animals are compared with experimental recipients of comparable numbers of non irradiated cells i.e. the 10^5 group, blood sugars, when measured, were always higher in the animals which had received irradiated cells, significantly so in 7 of 8 measurements ($p < 0.01$ in 6 measurements).

DISCUSSION

The study shows that streptozotocin induced diabetes mellitus can be passively transferred by transplants rich in T-lymphocytes, and that the number of cells required is small. Not only this, but within the confines of the study, smaller numbers of cells occasioned a more severe diabetic state in recipients than did larger numbers.

The fact, that a chemically induced diabetes can be transferred by a transplant comprised very largely of T-lymphocytes, supports the postulates of earlier studies that T-lymphocytes are important factors in the transfer of diabetes mellitus effected by transplant of spleen cells, and in the actual diabetogenic process. The direct significance of T-lymphocytes in passive transfer mechanisms has been demonstrated, also in other diseases, for example, experimental allergic orchitis (*Tung et al* 1977), arthritis (*Brackertz et al* 1977), contact sensitivity (*Zembala et al* 1977) and tuberculin hypersensitivity (*Tsuyuguchi & Kohmo* 1975)

Nude mice recipients also became diabetic, displaying blood glucose increases very similar to those seen in the comparable group of normal mice This suggests that functional thymus dependent lymphocytes in the recipient animal are not a prerequisite of the development of transfer diabetes, a conclusion which was also made after a comparable study with spleen cell transplants (*Buschard & Rygaard* 1977).

In this present study, both 10^3 and 10^4 cell groups display clearly diabetic blood sugar values These cell numbers are much lower than used in our earlier study of passive transfer of diabetes by transplant of spleen cells (4×10^7 cells – *Buschard & Rygaard* 1977), where only one cell quantity was studied.

A lesser number of T-lymphocytes (10^4) in the transplant was associated with higher blood sugars in the recipients than larger numbers ($10^6 - 10^7$). This was an unexpected finding, but the study has been repeated, and the results were similar. Maximum blood sugar of 242 ± 11 in the 10^3 group in this confirmatory study (obtained as early as the 13th day), was significantly higher than in other groups (10^4, 10^5, 10^6, 10^7).

For the further discussion an analysis of the composition of the inoculum is indispensable.

The initial treatment of the spleen cells a.m. *Böyum* (1968) gives a suspension containing approximately 80 per cent lymphocytes, 20 per cent monocytes and 1 per cent granulocytes.

The passage through the anti mouse Ig coated column removes cells bearing membrane immunoglobulin, i.e. B-lymphocytes (*Rubin & Wigzell* 1973). Likewise the monocytes must be removed by non-immune adherence to the beads of the column (*Mosier* 1976).

The remaining cells are thus non-B lymphocytes Of these, 85–94 per cent were killed with anti-theta and complement in the original experiments of *Rubin & Wigzell* (1973), leaving some 6–15 per cent of cells that were not sensitive to antitheta in the concentration used. We now know from the experiments of others that most or all of these are natural killer cells, NK-cells (*Kiessling et al.* 1975, *Herberman et al.* 1975). It is not known, and cannot be seen from our study, whether NK cells play any role in the diabetogenic process, but due to the extremely small numbers in the most active transplants, it seems highly unlikely. – This points to the T-lymphocytes.

All of the theta-sensitive cells by definition are T-lymphocytes It is not known, whether the procedure gives any selection of subpopulations of T-lymphocytes e.g. because of the presence of Fc receptors on T-cells, that might interact with the bound immunoglobulin on the column (*Katz* 1977) But it is here presumed that all T-lymphocyte subpopulations of the starting material of the spleen are represented, killer T, helper T and suppressor T cell groups

The relative roles of these populations are discussed in the following.

1 The T-killer cell is the obvious candidate to recognize and damage the beta-cell within the short period of time from transfer to appearance of signs of beta-cell insufficiency as witnessed by the increase in blood glucose values already after 3 days. Blood sugar values were fairly constant up to about the ninth day, which we interpret in terms of

one single initial damage during this first phase. The later upward movement in blood sugar values will be discussed later.

2. The involvement of a T-helper function, resulting in an antibody response of recipient B-lymphocytes in the initial phase is less probable. One argument is the short time between transfer and diabetes. Another consideration is the need for antigen if B-lymphocytes should be sensitised. It is not probable that enough donor beta-cell antigen could be transferred with the washed cell transplant. Recipient antigen might be released from beta-cells by an immediate cytotoxic effect of the T-killer cell. This might lead to a subsequent humoral response. Preliminary negative attempts to transfer diabetes with serum argue against such a humoral antibody mediated mechanism.

3. Some of the T-cells are suppressor cells. By definition they could suppress, but cannot induce the immunologic damage to the beta-cell. The high blood glucose values in the preliminary study of recipient mice receiving transplant of irradiated cells suggest the involvement of suppressor T-cells, because these cells are known to be relatively highly irradiation sensitive (*McCullagh* 1975, *Katz* 1977). If suppressor T-cells in the transplant are damaged while other cells are not affected to the same degree, a greater effect of the transplant might be expected.

The irradiation dose in our experiment – 200 R – was less than given by e.g. *McCullagh* (350 rad), but was measured by dosimetri at the irradiated object (naked cells), whereas McGullagh's was whole body irradiation of the experimental rats.

Suppressor T-cell function could also explain that recipients receiving more cells, e.g. 10^6 and 10^7, displayed lesser blood sugar increases than mice receiving fewer cells (10^3 and 10^4). The percentage of T-suppressor cells in the transplant is independent of transplant size but clearly the absolute quantity varies with transplant size. If it is assumed that it is the absolute quantity of T-suppressor cells which is critical for their suppressor function, this could explain the greater diabetogenic effect of the smaller transplant in the study.

It is possible that suppressor T-cells mediate their effects by a liquid product brought about in a higher concentration by a higher cell number. *Zembala et al* (1977) effected passive transfer of contact sensitivity by transplant of 4.4×10^7 mixed lymph node and spleen cells. The effect of the transplanted cells of the recipients could be inhibited if the transplants were first incubated with so called specific suppressor T-cell product. No dose-response curve was given in this study.

Related to the discussion of the role of suppressor T-cells in diabetogenesis is the study by *Jansen et al.* (1977) where mice given the diabetogenic encephalomyocarditis virus were treated with a cyclophosphamide derivative (Asta 5122). Another group of mice were treated with virus alone. Both groups displayed diabetic blood sugars on the seventh day, but mice which were treated with both virus and cyclophosphamide had significantly higher blood sugars than the mice which received only virus.

Röllinghoff et al. (1977) showed that T-suppressor cells are cyclophosphamide sensitive and this suggests that the findings in Jansen's study can be explained by cyclophosphamide suppression or destruction of T-suppressor cells, which entails that these cells no longer inhibit the T-killer (?) cells, cfr. 1. in this discussion.

A further indication that decrease in T-suppressor cell activity might be implicated in diabetogenesis stems from a study made by *Horowitz et al* (1977). These authors determined T-suppressor cell function in children with insulin dependent diabetes mellitus, and found, in six of nine children, that there was no T-suppressor cell function. Fifteen normal controls all had T-suppressor cell function.

A remarkable phenomenon in this present study was the pattern of development of diabetes. Blood sugar values were increasing relatively slowly and maximum values were reached first at the 20th–39th day. This pattern is different from that seen in transfer of diabetes by spleen cell transplant (*Buschard & Rygaard* 1977) when blood sugar maximum (268 ± 43) was seen after six days, thereafter to fall, (170 ± 34 on the 37th day – *Buschard* hitherto unpublished data). It should be mentioned, however, that the donor mice in this study displayed higher blood sugars, which may possibly be a salient factor.

In other transfer experiments recipients have also been affected at an early stage; after 4 days with experimental allergic orchitis (*Tung et al.* 1977), after two days with contact sensitivity (*Zembala et al* 1977), and after two days with allergic adrenalitis and encephalomyelitis (*Werdelin* 1972).

Two observations suggest some amplification mechanism in the present experiment, 1) the effect of smaller numbers of cells (10^3 & 10^4) and 2) the continuous upward movement in the blood sugar values up to the 20th–39th day. This secondary increase can depend on a donor T-helper cell effect on the recipient's immune apparatus, or on an 'autoimmune' process initiated by the recipient's immune apparatus after initial destruction of beta-cells by donor T-killer cells – that is to say that the destroyed beta-cells are regarded as »non self«.

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In conclusion, the study shows that streptozotocin induced diabetes mellitus can be passively transferred by transplants comprised very largely of

T-lymphocytes. Although the course of events and possibly the mechanism differs from that after passive transfer effected with spleen cells, the study again emphasizes a central role of thymus derived lymphocytes in the diabetogenesis.

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PURIFICATION OF THE FIRST COMPONENT OF GUINEA-PIG COMPLEMENT BY GEL CHROMATOGRAPHY

TORSTEIN LYBERG

Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3, Norway

Lyberg, T Purification of the first component of guinea-pig complement by gel chromatography. Acta path microbiol scand Sect C, *86*: 283–289, 1978

A method has been described for the purification of the first component (C1) of complement from guinea-pig serum The procedure consists in euglobulin precipitation followed by gel filtration on agarose columns The final product has low protein content and high specific activity. The protein obtained by this procedure has a molecular weight of about one million and has been further characterized using immunochemical and polyacrylamide gel electrophoresis techniques The protein reacts with anti C1 antiserum and forms the EAC1,4-intermediate The experiments indicated the existence of electrophoretic variants of C1 The dissociation of the C1 molecule by increasing the ionic strength is confirmed The described procedure appears to be a useful method of obtaining functionally purified C1

Key words Complement, C1 purification, gel chromatography, agarose

Torstein Lyberg, Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3, Norway

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The first component of complement (C1) exists in serum as a macromolecule with a molecular weight of about 10^6 daltons, which can be dissociated by EDTA into three major subcomponents, C1q, C1r and C1s The role of a fourth subcomponent, C1t, in the expression of C1 activity is disputed (2, 3, 10, 26)

The C1 macromolecular complex in human and guinea-pig sera can be prepared in a functionally pure form by euglobulin precipitation methods (23, 24), Sepharose chromatography (10, 25) and by sucrose gradient centrifugation procedures (8, 9) The most convenient procedure for isolation of C1 devised up to now is probably affinity chromatography using IgG as a ligand as described by Bing (4) and developed by *Assimeh et al* (1–3) and *Chapuis et al* (7)

We have investigated an isolation procedure for guinea-pig C1, combining the double precipitation method described by *Tamura & Nelson* (23) and gel filtration on agarose gels.

Since C1 is a considerable larger molecule than any other C component (sedimentation coefficient 19S), it was to expected that gel filtration offers a useful principle with respect to separation from C components and other serum proteins.

Gel filtration of C1 has earlier been performed on polyacrylamide gels (14) and on Sephadex G-200 (6, 13, 14, 22, 23). The recovery of C1 on Sephadex gels is low (14), probably because of the existence of anti-dextran antibodies in serum In addition, these gels have exclusion limits below the actual molecular weight of C1, and it seemed natural to apply the newer agarose gel chromatography media which have extended the molecular weight range of the gel chromatographic method. Agarose gels are considerably more rigid than polyacrylamide gels and therefore tolerate higher elution pressure, making it possible to obtain an adequate flow at 4° C, which is desirable because of the heat lability of C1

MATERIALS AND METHODS

Chemicals, Reagents and Antisera

The methods for the preparation of sheep erythrocytes (E) are described by Mayer (16, pp 149–150)

Guinea-pig blood was obtained by cardiac puncture of animals donated by the National Institute of Public

T-lymphocytes. Although the course of events and possibly the mechanism differs from that after passive transfer effected with spleen cells, the study again emphasizes a central role of thymus derived lymphocytes in the diabetogenesis.

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TABLE 1. *Purification of Cl by Double Precipitation at Low Ionic Strength and Chromatography on Bio-Gel A 5m*

Fraction	Volume[a]	Protein[b] mg/ml	Cl activity CH_{50} units/ml × 10^3	Specific activity CH_{50} units/mg protein × 10^3
Guinea-pig serum	1	44 000	69.4	1.577
0 04 M ppt	1	0.330	66.3	200.909
Re-ppt	0 25	0 255	214.8	842.352
Bio-Gel A 5m-pool	3	0 032	52 4	1637.500

[a] Original serum volume = 1
[b] Protein concentration was determined by the Folin method

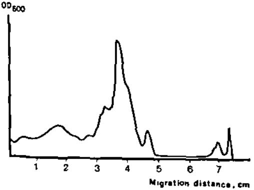


Fig 1 Scanning diagram of double-precipitated, partially purified Cl in 3 75 per cent polyacrylamide electrophoresis at pH 8 3 Migration distance measured from the negative to the positive electrode

was present in the first peak corresponding to the void volume.

The elution diagram of crude Cl on the same medium is shown in Fig. 3. Cl eluted in the same position, together with lipoproteins and other macroglobulins

Performing the chromatography of the crude preparation at increased ionic strength (μ = 0.60) gave separation of the bulk protein in the void volume, and Cl activity which was retarded on the column (Fig. 4).

In principle the same elution diagrams were obtained when chromatography was performed with Bio-Gel A 0 5m medium.

Gel Filtration on Bio-Gel A 5m

Since rather low yields were obtained when crude Cl was applied directly to an agarose column, the column was pretreated by passing 2 ml of whole guinea-pig serum through, followed by washing

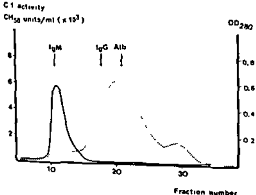


Fig 2 Elution diagram of whole guinea-pig serum after passage through a Bio-Gel A 1 5m column. Elution buffer VBS, μ = 0 15, containing Ca^{++} Human IgM, IgG and albumin were used as reference substances ——— = Cl activity, – – – – – = optical density at 280 nm

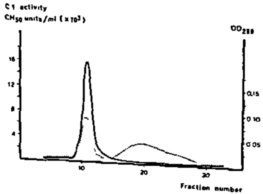


Fig 3 Elution diagram of partially purified Cl after passage through a Bio-Gel A 1 5m column. Elution buffer VBS, μ = 0 15, containing Ca^{++}, ——— = Cl activity, – – – – – = optical density at 280 nm.

Health (Oslo), serum was harvested, pooled and stored at −90°C.

Hemolysin was prepared by the method described by *Mayer* (16, pp. 150–151). It was used in a concentration twice the optimal level used in the whole complement titration (20, pp. 81–82).

Antiserum against Cl was produced by subcutaneous injections of partially purified Cl (A 1.5m pool) in rabbits. Rabbits received a dose of 0.5 ml (2 mg/ml) emulsified with an equal volume of Freund's complete adjuvant spread subcutaneously in the neck. The same procedure was repeated after 21 days, and rabbits were bled on the 7th day after the last immunization This antiserum contains antibodies directed against antigenic determinants accessible in the macromolecular form of Cl. No further characterization concerning the distribution profile of antibodies against the different subcomponents have been made. The antiserum agglutinated EACl but not EA cells.

Human and guinea-pig sera were used for calibrating the columns. The position of human IgM, IgG and albumin was monitored by radial immunodiffusion in agarose containing the respective antibodies obtained from Behringwerke AG, Frankfurt, W. Germany. Guinea-pig immunoglobulins were monitored by using rabbit antiserum against guinea-pig immunoglobulins also obtained from Behringwerke.

Veronal-buffered saline (VBS), pH 7.4, containing 0.001 M Mg^{++} and 0 00015 M Ca^{++}, 0.1 per cent gelatin and the ionic strengths 0.065, 0.15 and 0.60 respectively was made according to *Rapp & Borsos* (20, pp 75–78).

The 0.005 M phosphate buffer, pH 7.5, was made from two stock solutions containing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4

Gel Materials

Bio-Gel A 0 5m (100–200 mesh), Bio-Gel A 1.5m (100–200 mesh) and Bio-Gel A 5m (200–400 mesh) were obtained from Bio-Rad Laboratories, Richmond, CA, USA

Columns

For gel filtration, Pharmacia columns (1 5 × 30 cm) were used. Elution was performed at 4°C and usually at a flow rate of 30 ml/min.

Protein Preparations

Functionally purified guinea-pig C2 for the titration procedure was prepared by ammonium sulphate precipitation as described by *Nelson et al* (19).

Functionally purified Cl (crude Cl) for the preparation of EAC4 cells and for the further isolation procedure, was prepared according to the procedure devised by *Tamura & Nelson* (23). Briefly, serum was adjusted to pH 7.5 and diluted with water to ionic strength 0 04 The precipitate was washed once with VBS, μ 0.04, redissolved in isotonic VBS to the original volume and dialyzed against 0.005 M phosphate buffer, pH 7.5, for 4 h. The buffer was changed once during this period. The precipitate was once again washed in phosphate buffer and finally dissolved in isotonic VBS to one quarter of the original serum volume. The final preparation was clarified by centrifugation.

Assays

EAC4 cells for Cl titration were prepared and used as described by *Rapp & Borsos* (20, pp. 83–85).

Hemolytic titrations of Cl, C4, C2, and C3 were also done according to *Rapp & Borsos* (20, pp. 97–105)

Analytical Procedures

Isoelectric focusing. An LKB Ampholine column (LKB 8101) and carrier ampholyte solutions were obtained from LKB Products, Bromma, Sweden, and were used as described in the LKB 8100 Ampholine Electrofocusing Equipment Manual.

Ampholine carrier (2 per cent, pH 4–6) was used in the Cl electrofocusing experiments. The interior surface of the column was coated with a thin layer of polytetrafluoroethylene to minimize adhesion of glycoproteins to the walls (11). Electrofocusing was carried out at a constant voltage for 72 h. The temperature of the cooling system was maintained at 4°C. Fractions of 3.5 ml were collected by means of a peristaltic pump connected to the outlet tube of the column, and pH was determined at 4°C.

Disc electrophoresis. Analysis were carried out in a Buchler apparatus (Buchler Instruments Inc., NJ, USA) with glass columns (5 × 70 mm) The concentrating gel was 2.5 per cent acrylamide and the separating gel 3 75 per cent acrylamide. Tris/glycine buffer, pH 8.3, was used in the electrode compartments (15) The protein solution was mixed with an equal volume of 10 per cent sucrose, and 100μl of this mixture layered on top of the large-pore gel The gels were stained with 0 05 per cent Coomassie Brilliant Blue in methanol/acetic acid/water (45.20:9) for 30 min and destained in the same solution overnight. The gels were stored in 7 per cent acetic acid and scanned in a Joyce Loeble Chromoscan at OD_{600}

Immunoelectrophoresis and immunodiffusion analyses Immunoelectrophoresis was performed according to a modification of the method of *Scheidegger* (21), using 1 per cent agarose in tris/barbitone buffer, pH 8 6, which was also used for the double-diffusion analyses

RESULTS

Preparation of Crude Cl

Crude Cl was prepared as described under materials and methods Crude Cl preparations were purified several hundred folds over whole serum (Table 1), but contained small amounts of other complement components and large amounts of noncomplement protein. The disc electrophoresis pattern of crude Cl showed several bands (Fig. 1).

Gel Filtration on Bio-Gel A 1.5m and 0.5m

Gel filtration of whole guinea-pig serum on Bio-Gel A 1.5m (elution buffer VBS, μ = 0 15) showed three protein peaks as shown in Fig. 2. Cl activity

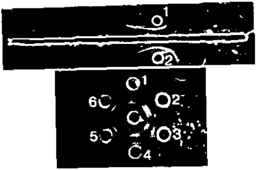


Fig 8 Immunoelectrophoresis and immunodiffusion of Cl Upper Immunoelectrophoresis of Cl. Well 1 contained the double-precipitated, crude Cl preparation and well 2 contained the purified Cl preparation. The trough contained rabbit anti-Cl-antiserum.
Lower Immunodiffusion of Cl Centre well, rabbit anti-Cl-antiserum Wells 1 and 2 contained GPS, wells 3 and 4 crude Cl and well 5 and 6 contained the concentrated, purified Cl preparation

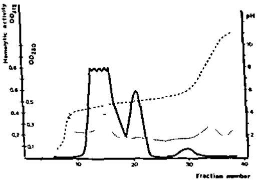


Fig 9 Isoelectric focusing of partially purified Cl on a 110-ml ampholine column, pH 4–6 ——— = Cl activity, - - - - - = optical density at 280 nm, ▪▪▪▪▪ = pH

activity could not be fully restored by reducing the ionic strength to 0 15 in the eluted fractions.

The most active fractions were pooled Assays for C4. C2 and C3 in the pooled fractions were carried out and found to be negative. The highly active fractions had a low protein content and the specific activity was more than 1500 CH_{50} units/µg protein

The pool of the most active fractions were concentrated on an Amicon Diaflow apparatus, Model 12, with a UM-10 membrane, and analyzed in a discontinuous polyacrylamide electrophoretic system Fig. 7 shows the scanned profile of the polyacrylamide gel, giving the appearance of two well-defined bands and some material which could not enter the separating gel.

The final product was also analyzed by immunodiffusion and immunoelectrophoresis (Fig. 8). By double-diffusion analysis against anti-Cl antiserum, a single precipitin line near the antigen well was seen with purified Cl preparations. By immunoelectrophoresis, two precipitin lines were detected. Purified Cl praparations gave no precipitin lines against anti-guinea-pig immunoglobulin antiserum.

The Cl nature of the final preparation was further assessed by the demonstration of neutralizing effect of the anti-Cl antiserum on the Cl hemolytic activity The purified preparations gave linear plots of relative Cl concentrations versus number of SAC'1a,4 generated per cell (5).

Isoelectric Focusing of Cl

Preliminary experiments indicated that Cl retained its hemolytic activity after being dissolved in a solution containing 2 per cent ampholyte (pH 4–6).

In the electrofocusing experiments, samples of 0.5–1.0 ml of crude Cl were substituted for the light ampholyte solution approximately in the middle of the gradient. Very little Cl activity was retained if the fractions were tested directly. After reconstitution of physiologic ionic strength and pH in the fractions, about 25 per cent of Cl hemolytic activity was recovered from the column Two well-defined peaks of Cl activity were found with pI values of 4.6 and 4.9 and, in addition, a minor peak at pI 5.6, but more than 95 per cent of the activity appeared within the peak with pI 4.6 (Fig. 9).

Isoelectric fractionation of the purified Cl preparation showed two hemolytic peaks at pI 4.6 and 4.9, but the yield was still lower, in the range of 5–10 per cent.

DISCUSSION

Chromatography of crude Cl on Bio-Gel A 0.5m and Bio-Gel 1.5m at isotonic elution conditions showed that Cl was eluted in the void volume together with inter alia lipoproteins, IgM and α-2-macroglobulin. This finding is in accordance with earlier gel chromatography studies on Cl using polyacrylamide (14) and Sephadex (6, 13, 22) media.

Chromatography of crude Cl on Bio-Gel A 5m gave still better resolution of containing proteins, and was preferred in the continuing experiments. At isotonic elution conditions, Cl was still contaminated with 19S macroglobulins, but free of lipoproteins and 7S and lower molecular weight substances.

Chromatography of crude Cl at high ionic strength (µ = 0.60) gave separation of Cl activity

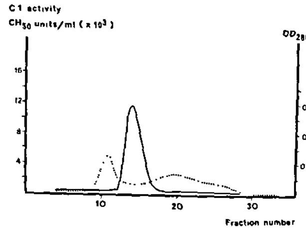


Fig. 4. Elution diagram of partially purified Cl after passage through a Bio-Gel A 1.5 column. Elution buffer: VBS, μ = 0.60, containing Ca + +. ——— = Cl activity; – – – – – = optical density at 280 nm.

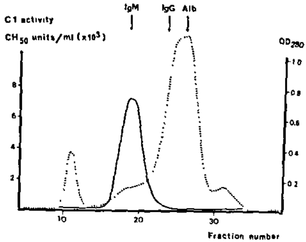


Fig. 5. Elution diagram of whole guinea-pig serum after passage through a Bio-Gel A 5m column. Elution buffer: VBS, μ = 0.15, containing Ca + +. Human IgM, IgG and albumin were used as reference substances ——— = Cl activity; – – – – – = optical density at 280 nm

with VBS, μ = 0.15, until effluent was free of protein. Fig. 5 shows the elution diagram of whole guinea-pig serum. Serum was separated into four definite peaks, with Cl activity positioned with the macroglobulin peak (inter alia IgM and α_2M).

Variability in the position of Cl was noted with various samples of sera. In some sera the main Cl activity eluted with the 7S protein peak. In our experience, this is most often seen with outdated sera. We have also, in some experiments, noted Cl activity in the void volume, which indicated aggregation of Cl molecules.

Chromatography of crude Cl on Bio-Gel A 5m (Fig. 6) separated Cl from lipoproteins which are excluded from the gel and from 7S and lower molecular weight proteins. Using VBS buffer, μ = 0.15, the macroglobulins still eluted in the same positions as Cl.

The separation of Cl from macroglobulins was established by increasing the ionic strength to μ = 0.60. Thereby Cl was retarded on the column in the same way as shown in Fig. 4, indicating splitting of the Cl molecule with increased ionic strength.

The recovery of Cl after elution with VBS, μ = 0.15, was 70–75 per cent of the activity applied, decreasing with prolonged use of the column. The recovery after elution at μ = 0.60 was about 70 per cent of the recovery after elution at μ = 0.15. The

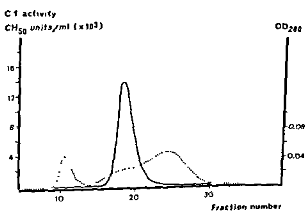


Fig. 6. Elution diagram of partially purified Cl after passage through a Bio-Gel A 5m column Elution buffer VBS, μ = 0 15, containing Ca + +. ——— = Cl activity; – – – – – = optical density at 280 nm.

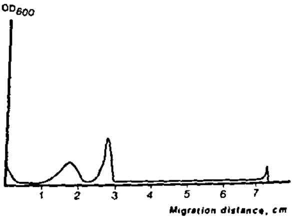


Fig 7 Scanning diagram of purified Cl (pooled, concentrated active fractions after gel filtration on Bio-Gel A 5m) in 3 75 per cent acrylamide electrophoresis at pH 8 3. Migration distance measured from the negative to the positive electrode.

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from other high molecular weight serum proteins on all the applied agarose media. This displacement of the Cl activity peak can only be explained by a splitting of the Cl molecule into smaller fragments by the increased ionic strength. This finding is in good accordance with the demonstration of Colten *et al.* (9) that the sedimentation coefficient of Cl varies inversely with the ionic strength. Prolonged storage of serum also seems to result in a dissociation of the Cl molecule.

The purified Cl preparation showed one precipitin line in double-diffusion (Ouchterlony) analysis against anti-Cl antiserum. By immunoelectrophoresis, two definite components were detected and by disc electrophoresis also two components which entered the separating gel and, in addition, some material which did not enter the running gel. These results agree well with those obtained by *Bing* (4) on human Cl, and suggests the existence of electrophoretic variants of human and guinea-pig Cl.

This finding was confirmed by isoelectric focusing of Cl which demonstrated two definite peaks with different pI values. The observed values correspond quite well with the values for Cl given by *Mukojima & Tachibana* (17), and can be taken as an indication for the existence of electrophoretic variants of macromolecular Cl. *Nelson & Brebner* (18) recovered Cl activity mostly at pH 4.95, but with a small »shoulder« of activity at pH 5.6.

Another possibility is that hemolytic activity is effected by Cl subcomponent complexes as described by *Laurell* et al. (12). It is possible that the electric forces acting upon the Cl molecule during electrophoresis changes the equilibrium position towards increasing concentrations of free subcomponents and complexes of these. Because of lack of antisera against purified preparations of guinea-pig Cl subcomponents, no indication is given in this study with respect to the composition of the electrophoretic variants.

The substantial loss of Cl activity in isoelectric focusing, for which we have no explanation, renders this method less suitable as a purification step for Cl.

It is concluded that the combination of euglobulin precipitation and gel filtration on Bio-Gel A 5m provide a useful mean for obtaining functionally purified Cl with little contamination by other proteins.

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NEUTRALIZING ANTIBODIES AGAINST HUMAN LEUKOCYTE, LYMPHOBLASTOID AND FIBROBLAST INTERFERONS ELICITED BY IMMUNIZATION WITH HUMAN LEUKOCYTE INTERFERON

LIISA PYHÄLÄ

Central Public Health Laboratory, Helsinki, Finland

Pyhala, L. Neutralizing antibodies against human leukocyte, lymphoblastoid and fibroblast interferons elicited by immunization with human leukocyte interferon. Acta path microbiol scand Sect. C, 86: 291–298, 1978.

Guinea pigs, rabbits and sheep were immunized with partially purified human leukocyte interferon. The antisera were tested for their neutralizing activities against human leukocyte, lymphoblastoid (Namalva) and fibroblast interferons. Rabbits and sheep developed high levels of neutralizing antibodies against all three interferons. The ratios of the neutralizing activities of leukocyte/lymphoblastoid interferons varied from 1 to 25 and those of leukocyte/fibroblast interferons from 2 to 500. The ratios increased with prolonged immunization. The guinea pigs developed relatively low levels of anti-leukocyte and anti-lymphoblastoid antibodies and as a rule failed to produce detectable neutralizing activity against fibroblast interferon. One sheep was given a booster injection of fibroblast interferon after preimmunization with leukocyte interferon. The booster injection stimulated a rise in antibody levels, not only for fibroblast interferon, but also for leukocyte and lymphoblastoid interferons. The antigenic relationships between leukocyte, lymphoblastoid and fibroblast interferons are discussed.

Key words: Human leukocyte interferon, human fibroblast interferon, human lymphoblastoid interferon, anti-interferon antibodies

Liisa Pyhala, Central Public Health Laboratory, Mannerheimintie 166, 00280 Helsinki 28, Finland.

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Human interferons derived from peripheral blood leukocytes and cultured diploid fibroblasts have been shown to have different physicochemical, antiviral and antigenic properties (7, 17, 23). Experiments using antibody affinity chromatography seem to suggest that there are two distinct antigenic species of interferon in human leukocyte interferon preparations, a major one specific for leukocyte interferon and a minor one shared by the interferon produced in fibroblasts (11, 19). This concept was confirmed recently by *Havell et al.* (13). They fractionated leukocyte interferon into four isoelectric components, one of which contained the antigenic specificity of fibroblast interferon. The cultured lymphoblastoid cells of the Namalva line have emerged as a potential cell source for the large-scale production of human interferon (4, 21, 22). *Havell et al.* (14) reported recently that Namalva interferon can also be fractionated by antibody affinity chromatography into two antigenic species of interferon. The main component is similar to the major antigenic species of leukocyte interferon and the minor one possesses the antigenic specificity of fibroblast interferon. The physicochemical and antiviral properties of Namalva interferon resemble those of leukocyte interferon (9, 10, 14, 21).

Human interferons are immunogenic in hetero-specific animals (17). Potent and specific anti-interferon sera are needed for the purification of interferons by antibody affinity chromatography and for the analysis of their various biological activities. Anti-leukocyte interferon globulins have proved to be useful in immunological purification of leukocyte, lymphoblastoid and fibroblast interferons (1–4).

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Human interferons are immunogenic in heterospecific animals (17). Potent and specific anti-interferon sera are needed for the purification of interferons by antibody affinity chromatography and for the analysis of their various biological activities. Anti-leukocyte interferon globulins have proved to be useful in immunological purification of leukocyte, lymphoblastoid and fibroblast interferons (1–4).

Large amounts of leukocyte interferon and long-term immunizations are needed to produce potent antisera (3, 6, 15, 18). The primary purpose of this work was to improve procedures for evoking potent anti-interferon sera and to provide information concerning the choice of animal species to be used for immunization. Guinea pigs, rabbits and sheep were immunized with leukocyte interferon and the antisera were compared with respect to their reactivities against leukocyte, lymphoblastoid and fibroblast interferons.

MATERIALS AND METHODS

Interferons. Human leukocyte interferon (Le-IF) was prepared by the Sendai virus induction of buffy-coat leukocytes and purified by selective precipitation from an ethanolic solution (17). Partially purified interferon preparation (P-IF) contained $1.0–1.5 \times 10^7$ units per ml and its specific activity was 5.0×10^5 units per mg protein.

The fibroblast interferon (F-IF) used for animal immunization was kindly provided by Dr. *R. Skoda* (Rentschler Laboratories, Laupheim, Federal Republic of Germany) and was produced in diploid foreskin cells by the superinduction schedule (12). Partially purified F-IF contained 4.0×10^5 units per ml and had a specific activity of 7.0×10^4 units per mg protein.

Crude human interferons used as antigens in neutralization tests were derived from peripheral leukocytes, cultured fibroblasts (FS-4) and lymphoblastoid cells (Namalva). F-IF induced by the superinduction method (12) was kindly provided by Dr *E. Mogensen* (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France). Namalva interferon (Na-IF) was prepared by Sendai virus induction (21) and was generously donated by Dr. *N. Finter* (Wellcome Research Laboratories, Beckenham, England). The infectivity of Sendai virus in crude Le-IF and Na-IF was destroyed by treatment at pH 2.

Interferon assay. Three-fold serial dilutions of interferons were assayed by the inhibition of vesicular stomatitis virus (VSV) plaques on human amnion (U) cells (20). All units are expressed in relation to the human reference preparation 69/19 (National Institute for Medical Research, Mill Hill, London). The dose response curves of crude Le-IF, Na-IF and F-IF showed similar slopes, when the plaque reduction was 20–70 per cent (8).

Neutralization assay. Three- or ten-fold serial dilutions of interferons were titrated against a fixed dilution of antiserum (16, 18). The neutralizing titre of antiserum was defined as the reciprocal of the interferon dose needed to reduce VSV plaques on U cells by 50 per cent in the presence of the antiserum. All titres of antisera are expressed in relation to the laboratory standard antiserum produced by immunization of sheep (»Iivar«) with human Le-IF (18). One ml of standard antiserum neutralized 30,000 units of Le-IF.

Immunization. Guinea pigs, rabbits and sheep were obtained from the random-bred animals at the Central Public Health Laboratory (Helsinki, Finland). Guinea pigs, weighing 500–600 g, received 16 weekly doses of 60,000 units of P-IF without adjuvant. Subsequently, two booster injections of 2.4 million units of P-IF with Freund's complete adjuvant (FCA) were given at an interval of 4 weeks. Rabbits, weighing 3.5–4.0 kg, received eight weekly doses of 3 million units of P-IF without adjuvant. Subsequently, the various boosters of P-IF with FCA were given as described in Fig. 1. Sheep, weighing 38–47 kg at the start and 69–76 kg at the end of the immunization period, received 10 weekly doses of 3 million units of P-IF without adjuvant. The various booster injections of P-IF with FCA were then given as described in Fig. 2.

All injections were given subcutaneously. P-IF for boosters was always emulsified with an equal volume of FCA. Bleedings were performed as described previously (18). Immune sera were heated at 56°C for 30 min and kept frozen at −20°C until titrated for antibodies to interferons.

RESULTS

Development of Neutralizing Antibodies

According to the efficient immunization schedule outlined previously (18), the P-IF injections were given initially without adjuvant and, after the rest period, with FCA. The initial immunization elicited the peak anti-Le-IF levels in the rabbits and sheep during the 8th and 9th weeks. The antibody titres were 15,000–30,000 in the rabbits and 900–1,500 in the sheep. This was in accordance with the dosage of P-IF per kg of body weight.

In order to ascertain the minimum booster dose of interferon capable of eliciting maximum anti-interferon activities, the rabbits and sheep received varying amounts of P-IF with FCA as indicated in Figs. 1 and 2. The antibody titres for Le-IF increased with the number of interferon units injected, up to 3 million units in the rabbits and 10 million units in the sheep. Ten million units of P-IF also elicited maximum responses to Na-IF and F-IF in the sheep, whereas the rabbits showed greater individual differences in their response to the heterologous interferons.

The late booster injections did not raise the anti-Le-IF levels beyond those brought about by the first optimal booster doses (Figs. 1 and 2). After the third boosters the peak responses to Na-IF and F-IF were only 10 to 70 per cent and 3 to 30 per cent of the highest responses elicited with earlier optimal boosters.

Ratios of Neutralizing Activities

The ratios of neutralizing activities against Le-IF and Na-IF are shown in Table 1. Maximum anti-Na-IF responses triggered by the first optimal boosters with P-IF were almost as high as the anti-

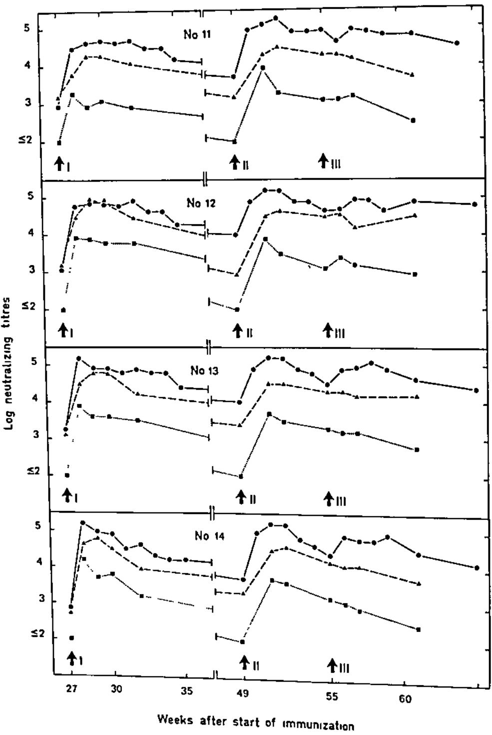


Fig 1 Antibody responses against Le-IF (●——●), Na-IF (▲– – –▲) and F-IF (■·····■) in rabbits nos. 11, 12, 13 and 14 immunized with P-IF The rabbits, preimmunized with eight weekly injections of 3 million units of P-IF without adjuvant, received booster injections I, II, III with FCA at the times indicated by the arrows. I- 0.3 million units to no 11, 1 million units to no 12, 3 million units to no 13, 10 million units to no 14; II and III 3 million units

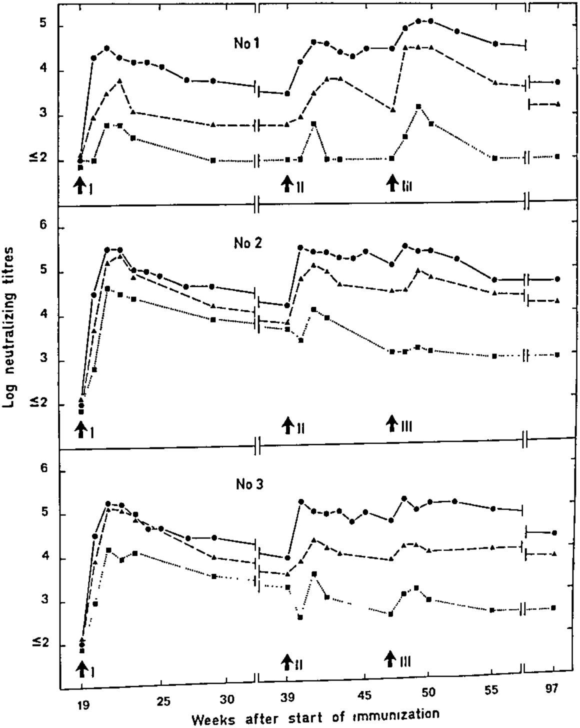


Fig. 2. Antibody responses against Le-IF (●——●), Na-IF (▲- - -▲) and F-IF (■····■) in sheeps nos 1, 2 and 3 immunized with P-IF. The sheep, preimmunized with 10 weekly injections of 3 million units of P-IF without adjuvant, received booster injections I, II, III with FCA at the times indicated by the arrows I 1 million units to no 1; 10 million units to no. 2; 100 million units to no 3, II and III 10 million units

Le-IF levels. However, during the course of the immunization the ratios of anti-Le-IF and anti-Na-IF levels tended to increase; the average ratio was about 5.

The ratios of anti-Le-IF level to anti-F-IF level are given in Table 2. After the first boosters the anti-Le-IF levels were on an average 20 times higher than the anti-F-IF levels After the third boosters there was roughly a 100-fold difference between the titres

TABLE 1. *Ratios of Neutralizing Activities against Le-IF and Na-IF in the Sera of Rabbits and Sheep Immunized with P-IF*

Animal		Anti-Le-IF / Anti-Na-IF					
		I		II		III	
		Mean	Range	Mean	Range	Mean	Range
Rabbit no	11	3	1–4	5	4–6	7	2–12
	12	3	1–11	3	1–5	3	1–6
	13	3	1–5	4	1–5	4	3–6
	14	3	1–4	4	2–5	6	5–6
Sheep no	1	12	4–23	13	4–25	5	3–7
	2	3	1–6	4	2–5	4	2–8
	3	2	1–4	11	5–25	9	3–13

I, II and III denote the boosters administered with FCA as described in the legends to Figs. 1 and 2. The ratios are calculated from the titres obtained after the indicated booster and shown in Figs 1 and 2. Each mean consists of three to six values

Persistence of Antibody Levels

After the boosters with P-IF the neutralizing activities against Le-IF, Na-IF and F-IF declined at about the same rates (Figs 1 and 2). Twenty weeks after the first boosters, the anti-interferon levels were at or below 10 per cent of the peak levels. The antibody titres decreased more slowly after the second and third boosters Thus, 48 weeks after the third boosters the anti-interferon levels were 13 to 60 per cent of the peak levels in sheep nos 2 and 3, which had received 60 and 150 million units of interferon during the whole immunization period.

Responsiveness of Different Animal Species

It can be seen from Table 3 that guinea pigs, rabbits and sheep differed in their responsiveness to P-IF The anti-Le-IF and anti-Na-IF levels attained in guinea pigs were about 10 per cent of those in rabbits and sheep. Only one guinea pig developed a detectable response to F-IF. The average anti-

TABLE 2 *Ratios of Neutralizing Activities against Le-IF and F-IF in the Sera of Rabbits and Sheep Immunized with P-IF*

Animal		Anti-Le-IF / Anti-F-IF					
		I		II		III	
		Mean	Range	Mean	Range	Mean	Range
Rabbit no	11	31	3–58	58	12–86	99	38–200
	12	14	4–33	36	20–50	54	19–83
	13	21	5–33	30	14–50	62	50–75
	14	14	2–25	27	16–39	76	63–100
Sheep no	1	43	10–70	86	50–125	112	17–300
	2	13	3–50	69	20–125	123	50–250
	3	14	5–33	203	30–500	151	67–250

For details, see legend to Table 1

TABLE 3. *Highest Anti-Interferon Titres for Le-IF, Na-IF and F-IF Attained in Different Animal Species by Immunization with P-IF*

Animal (n)	Amount of P-IF injected × 10⁶ units/kg	*Neutralizing activities against*		
		Le-IF	Na-IF	F-IF
Guinea pig (3)	10.00 (6.8–12)	15500 (6000–30000)	6000 (2400–15000)	<300 (<300–900)
Rabbit (4)	7.6 (7.0–8.5)	162000 (150000–180000)	56200 (30000–90000)	10000 (7500–15000)
Sheep (3)	1.0 (0 8–2.6)	186300 (120000–300000)	44600 (12000–240000)	10000 (1500–45000)

Immunization schedules are described in Materials and Methods and in the legends to Figs. 1 and 2. The geometric means together with the range are given for the injected interferon amounts and neutralizing titres attained The amount of P-IF injected represents the minimum dose needed to raise the peak antibody level.

interferon levels obtained in rabbits and sheep were about the same. Clearly fewer interferon units per kg of body weight were used for immunization of the sheep as compared with the rabbits. The ratios of neutralizing activities were roughly the same in the three animal species.

F-IF Booster after Immunization with P-IF

Sheep no. 2, preimmunized with P-IF (Fig. 2), received a booster of 2 million units of F-IF with FCA (Fig. 3). A rapid 50-fold rise in neutralizing activity against F-IF developed. The peak activity (45,000) was as high as the highest anti-F-IF level

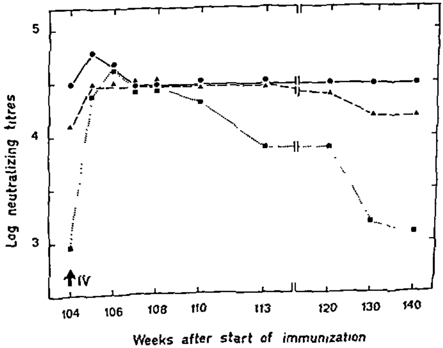


Fig. 3. Effect of the booster with partially purified F-IF on the antibody responses to Le-IF (●——●), Na-IF (▲- - -▲) and F-IF (■----■) in sheep no. 2. The initial immunization and boosters with P-IF are described in the legend to Fig 2. IV denotes the F-IF booster of 2 million units with FCA given at the time indicated by the arrow.

elicited earlier with P-IF. The booster with F-IF also evoked about a 2-fold increase in anti-Le-IF and anti-Na-IF levels. Neutralizing activity against F-IF did not decrease parallel with the anti-Le-IF activity. The slight decline in the anti-Na-IF level occurred simultaneously with the sharp fall in the anti-F-IF level.

DISCUSSION

In the present work immunizations were carried out with P-IF preparations of Le-IF. All the rabbits and sheep developed neutralizing activity against Le-IF, Na-IF and F-IF. The ratios of the neutralizing activities of Le-IF/Na-IF varied from 1 to 25. The cross-reactivity observed in the present work is in agreement with previous findings which indicated a close antigenic relationship between Le-IF and Na-IF (21). Neutralization of antiviral activity by monospecific antisera (5) and the binding to antibody affinity columns (14) were compatible with the concept that 60 to 90 per cent of the total activity of Na-IF is antigenically similar to the major component of Le-IF, and 10 to 20 per cent is a species of F-IF. In the present work the variability of the ratios for Le-IF/Na-IF suggests that the antigenic determinants of these interferons are not identical.

The ratios of neutralizing activities for Le-IF/F-IF varied in the present work from 2 to 500. This wide range agrees well with the results reported previously (3, 11, 19). About a 5-fold increase in the ratios was found in each of the animals during the course of the immunization. At the same time the neutralizing ratios for Le-IF/Na-IF increased to some degree. Thus, with the immunization process the antisera became increasingly specific towards the main antigenic component of Le-IF. It is worth noting that, as a general rule, a prolonged immunization is characterized by increasing avidity and decreasing specificity of the developing antibodies.

The F-IF booster after the prolonged immunization with Le-IF (sheep no. 2) strongly elevated the neutralizing activity against F-IF in the form of an anamnestic response. It also elicited a detectable rise in anti-Na-IF and anti-Le-IF levels. At least two hypotheses may be offered to explain the elevation of anti-Na-IF and anti-Le-IF levels. i) F-IF contains a minor antigenic component similar to the major component of Le-IF and Na-IF. This does not agree with the findings in earlier reports. The antisera raised against F-IF alone have failed to neutralize Le-IF (3, 11, 19). It should be noted, however, that these antisera have shown very low anti-F-IF activity as compared to the neutralizing activities attained in the present work. Indeed, the anti-F-IF levels elicited in sheep no. 2 are the highest ever reported. Potent antisera produced by immunization with Na-IF and with F-IF are needed to analyse the antigenic relationships between Le-IF, Na-IF and F-IF. ii) F-IF is antigenically monospecific and able to cross-stimulate the synthesis of neutralizing antibodies to the main antigenic component of Na-IF and Le-IF. The cross-stimulation which exists even in the late phase of the immunization process would suggest a close antigenic relationship, and would also explain the increased specificity of antisera obtained in the prolonged immunization with Le-IF.

In sheep no. 2 the decrease in the anti-F-IF and anti-Le-IF levels was not parallel after the booster with F-IF. This supports the concept, based on affinity chromatography studies, that the neutralizing activities against the two antigenic components of Le-IF reside in distinct antibody populations (11, 19).

Guinea pigs, rabbits and sheep differed in their responsiveness to P-IF. As far as the potency and the amount of antisera are concerned, the sheep seems to be the animal of choice. A low dosage of P-IF was sufficient for the initial immunization of the sheep, although it evoked anti-interferon levels that were barely measurable. However, the sheep proved to be sensitized sufficiently to respond well to boosters with FCA. In terms of the dosage of P-IF, the following guide can be suggested for the effective immunization of sheep. 8 to 10 weekly injections of 1×10^5 units per kg without adjuvant and a booster of 2.5×10^5 units per kg with adjuvant. Several studies are currently in progress at different laboratories to evaluate how useful the antisera produced in this work are for the purification of various human interferons by affinity chromatography.

The interferon preparations used in this study were obtained through the courtesy of Professor *Kari Cantell*, M.D. I am also grateful to Professor Cantell for his suggestions and help throughout this work, and to Professor *Kimmo Aho*, M.D. for his advice and criticism in the preparation of the manuscript. This study was supported by a grant from the *Finnish Cultural Foundation*.

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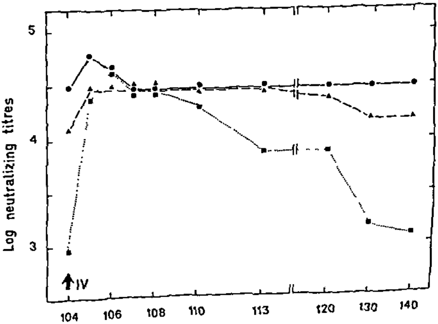


Fig. 3 Effect of the booster with partially purified F-IF on the antibody responses to Le-IF (●——●), Na-IF (▲– – –▲) and F-IF (■----■) in sheep no 2. The initial *immunization and boosters with* P-IF are described in the legend to Fig. 2 IV denotes the F-IF booster of 2 million units with FCA given at the time indicated by the arrow.

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FORMATION OF COMPLEXES COMPOSED OF $C\bar{1}r$, $C\bar{1}s$, AND $C\bar{1}$ INACTIVATOR IN HUMAN SERUM ON ACTIVATION OF C1

ANNA-BRITA LAURELL, U JOHNSON, U MÅRTENSSON and A G. SJÖHOLM

Institute of Medical Microbiology, Department of Immunology, University of Lund, Lund, Sweden

Laurell, A.-B., Johnson, U., Mårtensson, U & Sjoholm, A G Formation of complexes composed of $C\bar{1}r$, $C\bar{1}s$, and $C\bar{1}$ inactivator in human serum on activation of C1 Acta path microbiol scand Sect. C, *86* 299–306, 1978

C1 subcomponent complexes in normal serum incubated with C1q binding substances or with proteolytic enzymes were studied by crossed immunoelectrophoresis $C\bar{1}r$, $C\bar{1}s$, trypsin, heat aggregated IgG, immune complexes, cell-bound antibody or complexes of C-reactive protein with protamine sulphate gave rise to dissociation of C1 and the appearance of C1s protein in the α_2 position. In contrast, plasmin, DNA and endotoxin did not affect the normal C1s protein pattern Following incubation with $C\bar{1}r$, $C\bar{1}s$, trypsin, or complexes containing C-reactive protein, the C1q in serum was found in a cathodal position similar to that of purified C1q The C1s protein in the α_2 position was shown to be part of a complex composed of C1r, C1s and $C\bar{1}$ inactivator proteins C1r in the complex was only detected immunochemically by using anti C1r and anti C1s in combination. The complex had a molecular weight of about 5×10^5 daltons The results show that complexes of C1r, C1s and $C\bar{1}$ inactivator proteins in serum are formed on C1 activation, implying that the complexes contain activated C1r and C1s ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA) C1r-C1s complexes with β_1 mobility were not formed *in vitro* β_1 C1r-C1s complexes in pathological sera could be converted to $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA by treatment with $C\bar{1}r$ and $C\bar{1}s$ and also, provided C1q was available in serum, by treatment with heat aggregated IgG It was concluded that β_1 C1r-C1s complexes in serum contain C1r and C1s in proenzyme form

Key words C1 subcomponent complexes, C1 activation, crossed immunoelectrophoresis

A.-B Laurell, Institute of Medical Microbiology, Solvegatan 23, S-223 62 Lund, Sweden

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Macromolecular C1 is a Ca^{++}-dependent complex of C1q, C1r and C1s (20). On agarose electrophoresis with Ca^{++} in the gel medium, macromolecular C1 remains at the application site as can be demonstrated by crossed immunoelectrophoresis using monospecific antisera to C1q, C1r and C1s (15) Some pathological sera contain high amounts of C1r and C1s proteins not associated with C1q, and these yield C1s precipitates in the β_1 and α_2 regions on analysis by crossed immunoelectrophoresis (11, 15, 16) C1s with β_1 mobility was shown to be complexed with C1r, whereas the C1s in the α_2 region was found to be part of a complex composed of C1r, C1s and $C\bar{1}$ inactivator proteins (15) Since the $C\bar{1}$ inactivator reacts with $C\bar{1}r$ and $C\bar{1}s$ (8, 35), complexes of C1r, C1s and $C\bar{1}$ inactivator proteins in serum appear to reflect activation of C1 and may be designated $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA. The present investigation deals with the formation *in vitro* of C1 subcomponent complexes in normal serum using proteolytic enzymes and known activators of C1 such as aggregated IgG, immune complexes and complexes of C-reactive protein with protamine sulphate.

MATERIALS AND METHODS

Normal serum. Blood was drawn from apparently healthy donors and allowed to clot at room temperature for 1 hour. Aliquots of serum were kept at −80°C.

Sera containing C1r-C1s complexes C1q deficient

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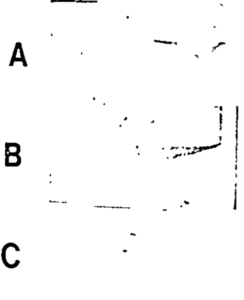


Fig 2 Crossed immunoelectrophoresis of C1q 0 15 M barbital buffer containing Ca^{++} in both electrophoretic steps
A Normal serum
B Normal serum incubated with $\overline{C1r}$
C Purified C1q
Arrow indicates application site Anode to the right.

about 60 per cent of the normal serum level of C1r as determined by electroimmunoassay Addition of $\overline{C1s}$ at less than 10 U per ml and of $\overline{C1r}$ at less than 5 per cent of the normal serum level of C1r did not appreciably affect the electrophoretic pattern of C1s

Incubation of normal serum with trypsin from 0 1 to 0 5 mg per ml similarly reduced the amounts of C1 at the application site and resulted in the appearance of C1s protein in the α_2 region and C1q in cathodal position In contrast, plasmin did not influence the C1s protein pattern in serum, even at high concentrations (7 5 CTA units per ml)

Heat aggregated IgG. Beginning at 0 1 mg per ml, aggregated IgG increased the α_2 C1s precipitate when incubated with normal serum at 37°C for 60 minutes Incubation of serum with IgG aggregates at 0 5 and at 1 mg per ml resulted in the gradual disappearance of C1r and C1s from the application site, associated with a shift of the C1s protein to the α_2 position (Fig 3A, B, C) C1q could not be detected by crossed immunoelectrophoresis, most likely due to binding of the protein to IgG aggregates

Immune complex formation Fifty µl aliquots of serum were mixed with 25 µl of antiovalbumin and 25 µl of ovalbumin and incubated at 37°C for 60 minutes. Ovalbumin was used at final concentrations ranging from 1 to 1000 µg per ml. Following incubation, the amounts of C1s protein in the α_2 position were increased over the whole range of ovalbumin concentrations. Antiovalbumin or ovalbumin added to serum alone did not influence the C1s protein pattern The amounts of C1s appearing in the α_2 region were greatest in the zone of far antigenic excess (1000 µg per ml) and in the zone of equivalence (5 µg per ml)

Cell-bound antibody. Formalinized sheep erythrocytes at 10^{10} per ml carrying various amounts of IgG or IgM antibody were incubated with serum at 37°C for 60 minutes. After incubation of serum with cells coated with the minimal agglutinating doses of antibody, the C1s protein pattern resembled that obtained with aggregated IgG at 1 mg per ml (Fig. 3 C) At approximately 25 per cent of the minimal agglutinating doses of IgG or IgM

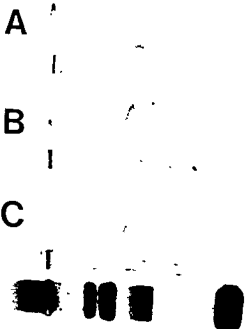


Fig 3. Crossed immunoelectrophoresis of C1s in serum Initial step in presence of Ca^{++}, second electrophoretic step in presence of EDTA.
A. Normal serum incubated at 37°C for 60 minutes
B Normal serum incubated with heat aggregated IgG (0.5 mg per ml)
C: Normal serum incubated with heat aggregated IgG (1 mg per ml).

serum from a patient with chronic urticaria (16) and serum from a patient with recurrent pneumococcal otitis media (11), were kept in aliquots at −80°C until analyzed.

Purification of complement components. C1q and C$\bar{1}$ inactivator (C$\bar{1}$ IA) were prepared according to published methods (26, 34). C1r protein was purified as described previously (15). The preparation contained no C1q or C1s assessed by immunochemical methods. Incubation with C1s resulted in ATEe hydrolysing activity, indicating that the C1r was in the active form, C$\bar{1}$r (23). The C1s used was prepared according to *Sakai & Stroud* (1973) and further purified by preparative agarose electrophoresis. C$\bar{1}$s was prepared according to *Haines & Lepow* (1964) and further purified by gel filtration on Sephadex G 200. The C$\bar{1}$s contained no C1r detectable by immunochemical methods.

Trypsin (2 × crystallized, Bovine pancreas, type III, Sigma, St. Louis 18, Missouri, U.S.A.) was dissolved in 0.0025 M hydrochloric acid at 10 mg per ml.

Human plasmin (KABI 1873, DtC 38, AB Kabi, Stockholm, Sweden) was used, containing 15 CTA units per ml as defined by *Johnson et al.* (1969).

Heat aggregated IgG. Commercial IgG (AB Kabi, Stockholm, Sweden) at 10 mg per ml, pH 6.5, was heated at 63°C for 30 minutes.

Immune complexes. Ovalbumin (Grade V, Sigma, St. Louis 18, U.S.A.) and the purified IgG fraction of rabbit antiovalbumin were used, containing 0.17 mg antibody per ml as determined by quantitative precipitation (12).

Cell-bound antibody. Formalinized sheep erythrocytes (33) were coated with rabbit IgM or IgG antibody. The minimal agglutinating doses were determined. The coated cells were washed 4 times before addition to serum.

C-reactive protein (CRP) was purified from pooled human serum or from ascitic fluid (*Johnson & Prellner* 1977).

Complexes of CRP with protamine sulphate were prepared as described by *Kaplan & Volanakis* (1974).

DNA (Deoxyribonucleic acid, Calf thymus, Worthington Biochemical Co., Freehold, N. Jersey, U.S.A.) and *Endotoxin* (Lipopolysaccharide W. E. coli 0 111 B4, Difco, Detroit, Michigan, U.S.A.) were used in physiological saline.

Antisera. Monospecific antisera to C1q, C1r, C1s, C$\bar{1}$ IA, and C-reactive protein were obtained as described earlier (10, 15, 27).

Electroimmunoassay (19) was employed for determination of C1q, C1r, C1s and C$\bar{1}$ IA (27, 30) and of IgG, IgM (5, 6) and CRP (10).

Crossed immunoelectrophoresis (18) of C1 subcomponents in serum was carried out as described previously (15). Complexes of CRP with protamine sulphate were also detected by crossed immunoelectrophoresis using anti CRP.

Gel filtration on Sepharose-CL 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was performed in a 2.5 × 100 cm column with 0.05 M acetate buffer pH 5.5, 0.2 M NaCl, 5 mM $CaCl_2$ (4). The column was calibrated by determination of IgG and IgM. Fractions were concentrated by pressure dialysis using a XM-50 membrane (Amicon B.V., Oosterhout /N.B./, Holland).

RESULTS

As described previously (15), analysis of the C1s protein in normal serum by crossed immunoelectrophoresis with Ca^{++} in the first and EDTA in the second electrophoretic step gave a sharp precipitate corresponding to macromolecular C1 at the application site and a minor precipitate in the α_2 region (Fig. 1 A). Using 0.15 M barbital buffer as applied for crossed immunoelectrophoresis of C1q (16), the C1q in serum appeared slightly cathodal to the application site (Fig. 2 A), together with C1r and C1s.

Proteolytic enzymes. Incubation of normal serum at 37°C for 15 minutes with C$\bar{1}$r (Fig. 1 B) or C$\bar{1}$s resulted in a decrease of the C1r and C1s associated with macromolecular C1 at the application site, and an increase of the α_2 C1s precipitate. C1q protein was found in the same position as purified C1q, cathodal to macromolecular C1 (Fig. 2 B, C). In these experiments, the C$\bar{1}$s was added at 100 U per ml as determined by hydrolysis of ATEe (14). C$\bar{1}$r was used in a final concentration corresponding to

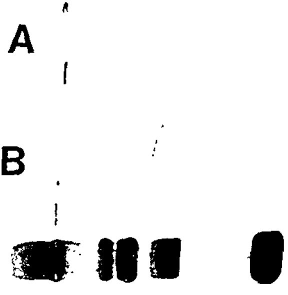


Fig. 1. Crossed immunoelectrophoresis of C1s in serum. Initial separation in presence of Ca^{++}, second electrophoretic step in presence of EDTA.
A. Normal serum incubated at 37°C for 15 minutes.
B. Normal serum incubated with C$\bar{1}$r at 37°C for 15 minutes.
Agarose electrophoresis of normal serum shown for reference.

serum were mixed with 25 µl of antiovalbumin and 25 µl of ovalbumin and incubated at 37°C for 60 minutes. Ovalbumin was used at final concentrations ranging from 1 to 1000 µg per ml Following incubation, the amounts of C1s protein in the α_2 position were increased over the whole range of ovalbumin concentrations Antiovalbumin or ovalbumin added to serum alone did not influence the C1s protein pattern. The amounts of C1s appearing in the α_2 region were greatest in the zone of far antigenic excess (1000 µg per ml) and in the zone of equivalence (5 µg per ml)

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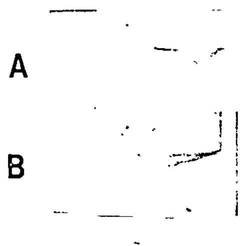


Fig 2 Crossed immunoelectrophoresis of C1q 0 15 M barbital buffer containing Ca ++ in both electrophoretic steps
A Normal serum
B Normal serum incubated with $\overline{C1r}$
C Purified C1q
Arrow indicates application site Anode to the right

about 60 per cent of the normal serum level of C1r as determined by electroimmunoassay Addition of $\overline{C1s}$ at less than 10 U per ml and of $\overline{C1r}$ at less than 5 per cent of the normal serum level of C1r did not appreciably affect the electrophoretic pattern of C1s

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Heat aggregated IgG Beginning at 0 1 mg per ml, aggregated IgG increased the α_2 C1s precipitate when incubated with normal serum at 37°C for 60 minutes Incubation of serum with IgG aggregates at 0 5 and at 1 mg per ml resulted in the gradual disappearance of C1r and C1s from the application site, associated with a shift of the C1s protein to the α_2 position (Fig 3A, B, C). C1q could not be detected by crossed immunoelectrophoresis, most likely due to binding of the protein to IgG aggregates

Immune complex formation Fifty µl aliquots of

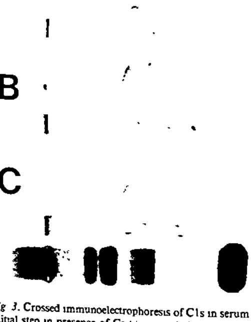


Fig 3. Crossed immunoelectrophoresis of C1s in serum Initial step in presence of Ca ++, second electrophoretic step in presence of EDTA
A Normal serum incubated at 37°C for 60 minutes
B Normal serum incubated with heat aggregated IgG (0 5 mg per ml)
C: Normal serum incubated with heat aggregated IgG (1 mg per ml)

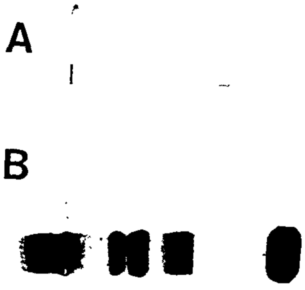


Fig. 4. Crossed immunoelectrophoresis of C1s in serum. Ca^{++} present in the first, EDTA in the second electrophoretic step.

A: Normal serum incubated with C-reactive protein (50 µg per ml) at 37°C for 30 minutes

B: Normal serum incubated with C-reactive protein (50 µg per ml) and protamine sulphate (200 µg per ml) at 37°C for 30 minutes.

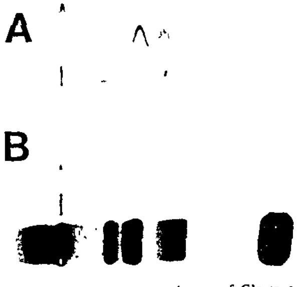


Fig. 5. Crossed immunoelectrophoresis of C1s in a pathological serum containing β_1 complexes of C1r and C1s and normal amounts of C1q.

A: Serum incubated at 37°C for 15 minutes.

B: Serum incubated with $C\bar{1}r$ at 37°C for 15 minutes. Ca^{++} in the first, EDTA in the second electrophoretic step

antibody, an increase of the α_2 C1s protein was still evident as compared to controls using unsensitized cells or serum incubated in the absence of cells.

CRP complexed with protamine sulphate. CRP was added to serum at 50 µg per ml and incubated with protamine sulphate at 37°C for 30 minutes. Protamine sulphate was used at 100 µg and at 200 µg per ml. With neither dose of protamine sulphate did C1q, C1r or C1s remain at the application site on crossed immunoelectrophoresis. The C1s protein was detected in the α_2 region (Fig. 4 A, B) and the C1q in a cathodal position. Protamine sulphate alone did not change the C1s protein pattern of normal serum. When protamine sulphate was used at 200 µg per ml, part of the CRP could be shown to be complexed with the substance as assessed by crossed immunoelectrophoresis using anti-CRP in the gel.

DNA and endotoxin. Incubation of serum at 37°C for 60 minutes with DNA (2 mg per ml) or endotoxin (0.8 mg per ml) did not appreciably affect the C1s protein pattern on crossed immunoelectrophoresis.

Pathological sera containing C1r-C1s complexes Two sera were studied, one with C1q within the normal range (Fig. 5 A) as determined by electroimmunoassay and one with C1q less than 1 per cent of the normal. Following incubation with $C\bar{1}r$ (Fig. 5 B) or $C\bar{1}s$, the serum with normal C1q showed a decrease of the C1s precipitate at the application site (Fig. 5 B). In both sera increased amounts of C1s protein appeared in the α_2 region, while no C1s remained in β_1 position

Incubation of the serum containing C1q with aggregated IgG gave rise to the same pattern as that shown in Fig. 5 B In contrast, incubation of the C1q deficient serum with aggregated IgG did not influence the C1s protein pattern Thus, treatment of serum with aggregated IgG only resulted in the disappearance of the C1r-C1s complexes when macromolecular C1 was available.

Gel filtration on Sepharose-CL 6B. Normal serum containing small amounts of β_1 C1r-C1s complexes was fractionated on Sepharose-Cl 6B. All the C1q, C1r and C1s, as determined by electroimmunoassay, emerged in fractions between the two main protein peaks (Fig 6). $C\bar{1}$ IA appeared in the main protein peak of the chromatogram together with IgG. Crossed immunoelectrophoresis of the concentrated fractions containing C1q, C1r and C1s showed the same C1s protein pattern as serum prior to gel filtration (Fig 7 A)

Normal serum incubated with aggregated IgG at 1 mg per ml, giving the C1s protein pattern shown in Fig. 3 C, was passed through the same column. Analysis of the fractions by electroimmunoassay

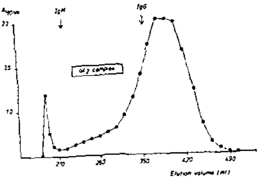


Fig 6 Gel filtration on Sepharose-CL 6B of normal serum incubated with heat aggregated IgG (1 mg per ml) at 37°C for 60 minutes Arrows indicate the maxima of the IgM and IgG peaks As determined by electroimmunoassay, complexes of C1r, C1s and C$\overline{1}$ inactivator proteins emerged with a maximum corresponding to an elution volume of 280 ml

gave diffuse C1s precipitates in fractions emerging between the IgG and IgM peaks, C1r was not detectable Addition of anti-C1r to anti-C1s in a gel containing Ca^{++} resulted in the appearance of sharply out-lined precipitates in the electroimmunoassay, demonstrating the presence of a complex containing at least C1r and C1s proteins On electroimmunoassay of the fractions with a mixture of anti-C1r, anti-C1s and anti-C$\overline{1}$ IA in the gel, the height of the C1r and C1s containing precipitates was markedly decreased Thus, the complex detected contained C1r, C1s and C$\overline{1}$ IA proteins. Most of the C$\overline{1}$ IA emerged in the main protein peak of the chromagram, indicating that only part of the C$\overline{1}$ IA in serum was complexed with C1r and C1s proteins No evidence was obtained to suggest that C1q was present in the complex Using IgM and IgG as references the complexes containing C1r, C1s and C$\overline{1}$ IA had a molecular weight of approximately 5×10^5 daltons

Fractions containing the complex of C1r, C1s and C$\overline{1}$ IA proteins were pooled and concentrated Analysis was carried out by crossed immunoelectrophoresis In the presence of EDTA in the second electrophoretic step, a precipitate in the α_2 region was obtained with anti-C1s that was similar to the α_2 precipitate in the serum prior to gel filtration (Fig 7 B) With Ca^{++} in both electrophoretic steps, anti-C1s gave a diffuse precipitate in the α_2 position (Fig 7 C) Addition of anti-C1r sharpened the outline and decreased the height of the precipitate (Fig. 7 D) Analysis with a mixture of anti-C1r, anti-C1s and anti-C$\overline{1}$ IA gave an α_2 precipitate that was further decreased in height (Fig. 7 E). Thus, the complexes found on *in vitro* activation of C1 in serum showed the same reaction patterns as the C$\overline{1}$r-C$\overline{1}$s-C$\overline{1}$ IA complexes previously described in some pathological sera (15).

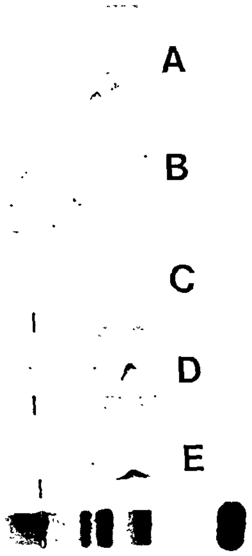


Fig 7 Crossed immunoelectrophoresis of pooled and concentrated fractions containing C1s after gel filtration of serum on Sepharose-CL 6B

A Normal serum applied to column
B–E Normal serum incubated with aggregated IgG applied to column
A–B Initial separation in presence of Ca^{++}, second electrophoretic step in presence of EDTA
C–E Ca^{++} present in both electrophoretic steps
A–C Anti-C1s in the gel
D Anti-C1s combined with anti-C1r in the gel
E. Anti-C1s combined with anti-C1r and anti-C$\overline{1}$ inactivator in the gel

DISCUSSION

The present investigation showed that substances capable of activating C1, and proteolytic enzymes such as C$\bar{1}$r, C$\bar{1}$s and trypsin, gave rise to complexes containing C1r, C1s and C$\bar{1}$ IA proteins in normal serum. These complexes appeared after incubating the serum with heat aggregated IgG or cell bound antibody, and on the formation in serum of immune complexes or complexes of CRP with protamine sulphate. On the other hand, C1q binding substances such as DNA and endotoxin (22, 31) did not give rise to complexes of C1r, C1s and C$\bar{1}$ inactivator proteins.

On crossed immunoelectrophoresis, the complexes of C1r, C1s and C1 $\bar{I}$A proteins formed *in vitro* were found in the α_2 region and showed the same characteristics (Fig. 7) as the C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes previously described in normal and pathological sera (15). The findings establish that α_2 complexes of C1r, C1s and C1$\bar{I}$A in serum signify activation of C1 as proposed earlier (15).

Following activation of C1 by proteolytic enzymes, or by CRP complexed with protamine sulphate, the C1q was shown on crossed immunoelectrophoresis to be located in a similar cathodal position to that of purified C1q. In experiments using heat aggregated IgG, the C1q was probably bound to the aggregates, and was therefore not detected by this method. Evidently, activation of C1 in serum resulted in dissociation of macromolecular C1 into C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes and C1q protein. The present study did not decide whether binding of C1r and C1s protein to C$\bar{1}$ IA precedes the dissociation of C1 or not. The observation that C1r becomes antigenically silent on activation of C1 (3) is in accordance with our earlier findings (15, 16) and with the data given in the present investigation.

β_1 complexes of C1r and C1s in pathological sera disappeared on activation of C1 by proteolytic enzymes or by aggregated IgG However, in C1q deficient serum the C1r-C1s complexes were not affected by treatment with aggregated IgG. The findings provide further evidence that the C1r and C1s in the β_1 complexes are in proenzyme form (15, 16)

On Sepharose-CL 6B gel filtration of serum treated with IgG aggregates, C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes showed an apparent molecular weight of about 5×10^5 daltons. This is in agreement with the previous finding of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes in the macromolecular fraction on Sephadex G 200 (15). The precise structure of the complex is not known.

Analysis by ultracentrifugation has provided some evidence that C$\bar{1}$ in serum and C$\bar{1}$ prepared with purified reagents are unstable (1, 3). By electrophoresis on polyacrylamide gels complexes of C$\bar{1}$s and C$\bar{1}$ IA were demonstrated following incubation of C$\bar{1}$ with serum (1). However, C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes were not identified with the experimental design used in these studies

It has been proposed that the detection in normal sera of α_2 C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes and β_1 complexes of C1r and C1s by crossed immunoelectrophoresis might be due to changes in the equilibrium of C1 subcomponents during electrophoresis (1, 37). In our experience, β_1 complexes are highly reproducible findings when present in serum On serial sampling over long periods, small amounts of β_1 complexes occur transiently in apparently healthy individuals, also when measurements by electroimmunoassay indicate equimolar concentrations of C1q, C1r and C1s in serum. Furthermore, the present recovery of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA and C1r-C1s complexes on gel filtration of normal serum seems to support the view that these complexes were preformed in the serum. It is on the other hand quite possible that the experimental conditions used in crossed immunoelectrophoresis favour the detection of small amounts of C1r-C1s complexes and C$\bar{1}$ IA bound to C1r and C1s proteins which may be difficult to detect by other means. The high sensitivity of the single radial immunodiffusion method described by *Ziccardi & Cooper* (1978) for added C1s might not apply when the C1s protein is complexed, explaining their failure to demonstrate »free C1s« in normal sera.

It is well established that C$\bar{1}$r activates purified C1s (23, 35) and is involved in the activation of macromolecular C1 (36). According to recent evidence autocatalytic activation of C1s (21) does not occur (32). Plasmin and trypsin have also been reported to activate purified C1s (24, 32).

Thus, available information as to the activation of C1 subcomponents by proteolytic enzymes does not clearly explain why C$\bar{1}$r, C$\bar{1}$s and trypsin caused activation and dissociation of macromolecular C1 in serum. Further work is required to define the molecular changes of C1 subcomponents associated with C1 activation by proteolytic enzymes. The conclusion from the present work that plasmin does not activate C1 in serum finds support in previous studies on the activation of complement by streptokinase (14) and on the cleavage of C4 by proteolytic enzymes (29).

Increased amounts in serum of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes and/or C1r-C1s complexes have been found in pathological conditions with an immunological back-ground, suggesting involvement of the complement system also in the absence of manifest hypocomplementemia (2, 11, 15, 16, 28) Measure-

ment of C1 subcomponent complexes may be of value in the assessment of some immunological diseases. A method for quantitative estimation of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes by electroimmunoassay has been devised (17).

The results of the present investigation suggest that activation of C1 by circulating or tissue bound immune complexes, CRP-containing complexes (13), or by proteolytic enzymes may account for the formation of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes in disease. On the other hand, the experiments described did not explain the occurrence of C1r-C1s complexes in serum.

C1 activation in normal serum suggested by the presence of small amounts of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes may be part of processes by which complement activation continuously takes place. If so, the importance of regulators of complement activation under physiological conditions is clearly evident.

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DISCUSSION

The present investigation showed that substances capable of activating C1, and proteolytic enzymes such as $C\overline{1r}$, $C\overline{1s}$ and trypsin, gave rise to complexes containing C1r, C1s and $C\overline{1}$ IA proteins in normal serum. These complexes appeared after incubating the serum with heat aggregated IgG or cell bound antibody, and on the formation in serum of immune complexes or complexes of CRP with protamine sulphate. On the other hand, C1q binding substances such as DNA and endotoxin (22, 31) did not give rise to complexes of C1r, C1s and $C\overline{1}$ inactivator proteins.

On crossed immunoelectrophoresis, the complexes of C1r, C1s and C1 $\overline{I}$A proteins formed *in vitro* were found in the α_2 region and showed the same characteristics (Fig. 7) as the $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes previously described in normal and pathological sera (15). The findings establish that α_2 complexes of C1r, C1s and C1$\overline{I}$A in serum signify activation of C1 as proposed earlier (15).

Following activation of C1 by proteolytic enzymes, or by CRP complexed with protamine sulphate, the C1q was shown on crossed immunoelectrophoresis to be located in a similar cathodal position to that of purified C1q. In experiments using heat aggregated IgG, the C1q was probably bound to the aggregates, and was therefore not detected by this method. Evidently, activation of C1 in serum resulted in dissociation of macromolecular C1 into $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes and C1q protein. The present study did not decide whether binding of C1r and C1s protein to $C\overline{1}$ IA precedes the dissociation of C1 or not. The observation that C1r becomes antigenically silent on activation of C1 (3) is in accordance with our earlier findings (15, 16) and with the data given in the present investigation.

β_1 complexes of C1r and C1s in pathological sera disappeared on activation of C1 by proteolytic enzymes or by aggregated IgG. However, in C1q deficient serum the C1r-C1s complexes were not affected by treatment with aggregated IgG. The findings provide further evidence that the C1r and C1s in the β_1 complexes are in proenzyme form (15, 16).

On Sepharose-CL 6B gel filtration of serum treated with IgG aggregates, $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes showed an apparent molecular weight of about 5×10^5 daltons. This is in agreement with the previous finding of $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes in the macromolecular fraction on Sephadex G 200 (15). The precise structure of the complex is not known.

Analysis by ultracentrifugation has provided some evidence that $C\overline{1}$ in serum and $C\overline{1}$ prepared with purified reagents are unstable (1, 3). By electrophoresis on polyacrylamide gels complexes of $C\overline{1s}$ and $C\overline{1}$ IA were demonstrated following incubation of $C\overline{1}$ with serum (1). However, $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes were not identified with the experimental design used in these studies.

It has been proposed that the detection in normal sera of α_2 $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA complexes and β_1 complexes of C1r and C1s by crossed immunoelectrophoresis might be due to changes in the equilibrium of C1 subcomponents during electrophoresis (1, 37). In our experience, β_1 complexes are highly reproducible findings when present in serum. On serial sampling over long periods, small amounts of β_1 complexes occur transiently in apparently healthy individuals, also when measurements by electroimmunoassay indicate equimolar concentrations of C1q, C1r and C1s in serum. Furthermore, the present recovery of $C\overline{1r}$-$C\overline{1s}$-$C\overline{1}$ IA and C1r-C1s complexes on gel filtration of normal serum seems to support the view that these complexes were preformed in the serum. It is on the other hand quite possible that the experimental conditions used in crossed immunoelectrophoresis favour the detection of small amounts of C1r-C1s complexes and $C\overline{1}$ IA bound to C1r and C1s proteins which may be difficult to detect by other means. The high sensitivity of the single radial immunodiffusion method described by *Ziccardi & Cooper* (1978) for added C1s might not apply when the C1s protein is complexed, explaining their failure to demonstrate »free C1s« in normal sera.

It is well established that $C\overline{1r}$ activates purified C1s (23, 35) and is involved in the activation of macromolecular C1 (36). According to recent evidence autocatalytic activation of C1s (21) does not occur (32). Plasmin and trypsin have also been reported to activate purified C1s (24, 32).

Thus, available information as to the activation of C1 subcomponents by proteolytic enzymes does not clearly explain why $C\overline{1r}$, $C\overline{1s}$ and trypsin caused activation and dissociation of macromolecular C1 in serum. Further work is required to define the molecular changes of C1 subcomponents associated with C1 activation by proteolytic enzymes. The conclusion from the present work that plasmin does not activate C1 in serum finds support in previous studies on the activation of complement by streptokinase (14) and on the cleavage of C4 by proteolytic enzymes (29).

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LYMPHOCYTE SUBPOPULATIONS IN MAN. EXPRESSION OF HLA-DR DETERMINANTS ON HUMAN T CELLS IN INFECTIOUS MONONUCLEOSIS

H E. JOHNSEN, M. MADSEN, T. KRISTENSEN and F. KISSMEYER-NIELSEN

The Tissue Typing Laboratory, University Hospital, DK-8000 Aarhus C, Denmark

Johnsen, H E, Madsen, M., Kristensen, T & Kissmeyer-Nielsen, F Lymphocyte suppopulations in man Expression of HLA-DR determinants om human T cells in infectious mononucleosis Acta path microbiol scand Sect C, 86 307–314, 1978

In a study of lymphocyte subpopulations in peripheral blood during infectious mononucleosis the lymphocytosis was found to be of T-cell origin (i e E-RFC), while the number of non T lymphocytes (i e EA-, EAC-RFC and SmIg + ve cells) was normal in 7 out of 8 patients Ten patients were tested for the presence of HLA-DR determinants on their B and T cells and not only B lymphocytes but also a great part (31–75%) of T cells were lysed by the anti HLA-DR testsera, indicating that HLA-DR determinants are expressed on a population of T cells in IM patients. After recovery all patients were retested and showed a normal pattern of HLA-DR typing This indicates an increase of HLA-DR antigens on T cells or a vigorous proliferation of a small DR-positive T-cell subpopulation during the acute stage of IM

Key words HLA-DR, T cells, infectious mononucleosis

H E Johnsen, The Tissue Typing Laboratory, University Hospital, DK-8000 Aarhus C, Denmark.

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Studies of membrane markers on peripheral blood lymphocytes during the acute phase of infectious mononucleosis (IM) have revealed that principally T- but also B-cells are involved in the lymphocytosis (5, 6, 19, 29) The Epstein-Barr virus (EBV), which is supposed to be the ethiological agent of IM, can infect human lymphocytes *in vitro*, establishing lymphoblastoid cell lines of B cell origin defined by membrane markers as well as by function (7, 22, 23, 32) The B lymphoblasts are EBV nuclear antigen (EBNA) positive, and this antigen can be used as an early valid marker for EBV infected lymphocytes (31) In peripheral blood from IM patients only a few EBNA positive blastoid cells (0 5–2%) have been found in the non-T-cell fraction, and none in the T-cell fraction (14)

The hypothesis of the pathogenesis in IM is that EBV infects only normal B lymphocytes with the specific virus receptor (7, 11, 31) converting them into cells with a potential for unlimited proliferation. These cells contain EBV genetic material, which results in EBV-determinated membrane antigens (MA) on the lymphoblasts These antigens are thought to be targets for a regulating immunological response by B- and T-cells, which in concert control the infection and the EBV genome responsible B-cell proliferation, hence reverting IM to a benign self-limiting disease (1, 4).

Cellular immunity during the acute stage is suggested by the presence of cytotoxic T-lymphocytes, specific for EBV genome positive targets (25, 26) which would stop the proliferation *in vivo* by actually destroying the transformed cells. Antibodies against MA have been proposed to be important in the subsequent control of the persisting latent EBV infection (12).

Another attractive mechanism is that B-cell

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HLA workshop 1977 (2). The T-cell suspension (E-RFC enriched) and the B-cell suspension (E-RFC depleted) were tested in parallel by a selected set of workshop VII antisera defining six DRw specificities Each specificity was defined by a minimum of two testsera; *DRw1*. sera Nos. 09, 128, 152, *DRw2*· sera Nos. 14, 16, 17, 18, 21, 60, 81; *DRw3* sera Nos 24, 25, 26, 27, 34, 35, 95, 133, 180, *DRw4*. sera Nos 37, 38, 104, 106, 129, *DRw7*. sera Nos 51, 52, 53, 54, 72; *DRw8*· sera Nos. 58, 112 (2)

Absorption experiments 100 µl of serum No. 054 (DRw7), which was positive with two of the patients' T and B cells, was mixed with an equal number (30 × 10[6] cells / 100 µl serum) of T cells from the patient and a normal DRw7 positive individual, respectively The mixture was then incubated first at 20° C for 30 minutes and then at 37° C for 30 minutes. The reactivity of the absorbed serum was tested against T and B cells from the patient and the normal person

RESULTS

Lymphocyte Subpopulations Quantitated by Membrane Markers

Serial quantitations of subpopulations in peripheral blood were conducted on 8 patients with IM. Table I shows that all patients had an initial leucocyte and lymphocytosis. The relative numbers of E-RFC in the 1st, 2nd and 3rd sample show no significant difference compared to the normal values. On the other hand the percentages of EA- and EAC-RFC were significantly decreased in the 1st and 2nd sample compared to the 3rd sample and the normal values. The percentages of SmIg positive lymphocytes were not significantly altered during the investigation.

The number of cells phagocytozing latex particles

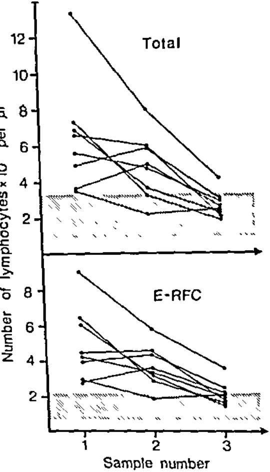


Fig I Serial quantitations of total number of lymphocytes and E-RFC in peripheral blood on patients with IM The hatched area is normal range values from normal donors

TABLE I *Lymphocyte Subpopulations in Peripheral Blood from 8 Patients during IM*

Sample number		Leucocytes per µl (× 10³)	Lymphocytes per µl (× 10³)	E-RFC %	EA-RFC %	EAC-RFC %	SmIg + ve %	Yield %
	mean	10 1	6 5	79 4	14 0	5.3	9 0	49.3
	range	7 4–19 6	3.5–13.3	68–92	10–25	02–09	05–32	38–67
	SD	4 1	3.1	8 6	5.2	2.5	2 9	10 6
1 week)	mean	7 5	4 8	78 6	15 4	4 4	9 3	53.9
	range	4 4–12 0	2 2–8 0	70–91	08–27	01–09	05–12	28–85
	SD	2 7	1 8	8 3	7 2	2 6	1 8	17.9
–14 week)	mean	6 6	2 8	79 8	20 3	13.6	10 7	46 3
	range	5 5–8 1	1 9–4 2	69–87	13–24	08–25	06–23	24–63
	SD	1 0	0 7	7 5	3 7	5 1	6 7	15 9
Normal values (= 35)	mean	6 65	2 1	71 0	22 1	11 5	17 3	52.2
	range	4 1–8 9	1 0–3 4	61–82	08–32	06–19	06–31	14–86
	SD	2 0	1 0	6.7	7 4	3 7	7 5	19.5

proliferation is controlled by the presence of suppressor T-lymphocytes as suggested by *Thorley-Lawson et al.* (28). Indeed, there are experimental data in man, which describe a propably non-cytotoxic inhibition of the *in vitro* outgrowth of EBV transformed lymphoblastoid cells, which is related to T cells from IM patients and previously infected persons (21, 28). If human suppressor cells exist they may be a subpopulation of T-cells with human Ia-like or HLA-*D* related (HLA-DR) antigens on the surface, in analogy to the suppressor T-cells defined in mice, which abrogates the B cell proliferation and differentiation. The responsible subpopulation has been identified with Ly-2,3, and Ia surface antigens (8, 17, 20).

The human Ia-like or HLA-DR antigens are serologically defined and coded for by the major histocompatibility complex (MHC) of man, and they are closely associated with or even identical to the D-locus antigens identified by mixed lymphocyte culture (MLC) typing (2). HLA-DR antigens are the human counterpart to the serological defined immune response associated (Ia) antigens in mice coded for in the central I region of the H-2 complex. In contrast to the classical HLA-A, B and C antigens present in the membrane of all lymphocytes, the HLA-DR antigens are predominantly expressed and defined on B-lymphocytes, while T-lymphocytes isolated from peripheral blood do not present detectable HLA-DR antigens on their surface.

In view of the hypothesis concerning suppressor cells in IM we decided to investigate whether HLA-DR antigens were present on IM T-cells.

MATERIALS AND METHODS

Patients Ten patients (age 11–30 years, ♂/♀ 8/2) with the diagnosis IM were selected on the basis of clinical features, relative and/or absolute lymphocytosis in peripheral blood including atypic lymphocytes and the occurrence of a positive test for heterofile agglutinins (Paul-Bunnel or Monospot tests).

Blood samples were obtained from the patients during the first fortnight after the onset of disease Eight patients were retested during the following week and in the reconvalescence period 4–14 weeks after the onset of symptoms Two patients, who could not be reinvestigated, were excluded from the study of lymphocyte subpopulations.

Isolation of mononuclear cells Defibrinated blood was used as previously described (9). Cells were isolated ad modum *Bøyum* (3) with local modifications, the blood, diluted 1 2 in BSS, was layered on Lymphoprep® (Nygård & Co A/S, Oslo) centrifuged with 1,000 G at the interphase for 20 minutes. Mononuclear cells were harvested and washed twice in BSS (500 G for 10 minutes) and adjusted to 3×10^6 cells per ml BSS. The total lymphocyte yield during the isolation procedure was calculated for all suspensions.

Phagocytes were identified by the latex phagocytose test (30): two ml of the cell suspension was mixed with 100 µl of a 1% (v/v) latex suspension (diameter 0 81 µm) and 2 ml fetal calf serum (FCS). After incubation at 37° C for 30 minutes the cells were washed twice and resuspended in BSS. This suspension was used for the further identification of lymphocyte membrane markers.

Quantitation of E-rosette forming cells (E-RFC) was performed as previously described (15). Briefly, sheep red blood cells (SRBC) were treated with 0.14 M 2-aminoethylisothiouronium bromide (AET) (Sigma), washed in saline and resuspended to 1% (v/v) in BSS with 40% fetal calf serum (FCS). Duplicate tubes with 100 µl of lymphocyte suspension with 100 µl AET-treated SRBC suspension were incubated at 37° C for 15 minutes, then centrifuged at 45 *g* for 5 minutes, and incubated overnight at 4° C. After toluidine blue staining and gentle resuspension, rosettes were scored in a Burker-Turk haemocytometer; counting lymphocytes with 5 or more SRBC attached as rosettes. A total of 200 lymphocytes were counted.

Quantitation of erythrocyte antibody- (EA) and erythrocyte antibody complement- (EAC) RFC was performed as previously described (10). A 1% suspension (v/v) in BSS with 10% FCS of EA and EAC indicator cells was prepared after sensitization of ox erythrocytes (ORBC) with a hyperimmune rabbit anti-ORBC serum (serum A7) and a zymosan-treated normal human serum (»R3«), respectively. The A7 serum contains anti-ORBC antibodies of the IgG class, while the »R3« serum contains antibodies of the IgM class and is deficient in C5 and hence nonhaemolytic.

The rosette tests were performed in duplicate by mixing 100 µl of the mononuclear cell suspension and 100 µl of the indicator cells, after centrifugation at 45 *g* for 5 minutes the test tubes were incubated at 37° C for 15 minutes, followed by 4° C overnight. Enumeration of RFC was done after toluidine blue staining, counting a total of 200 lymphocytes.

Quantitation of SmIg-positive lymphocytes SmIg-positive cells were enumerated by a slightly modified direct immunofluorescense technique (18) In brief, 1–2 $\times 10^6$ lymphocytes were incubated at 4° C for 30 minutes in 100 µl FITC-conjugated polyvalent rabbit antihuman Ig (Dakopatts, Denmark) diluted 1 10 After two washes scoring was performed in a fluorescense microscope

Fractionation of E- and non-E-RFC Briefly, 3 ml lymphocyte suspension was mixed with 3 ml of 1% AET-treated SRBS in 40% FCS, centrifuged at 50 *g* for 5 minutes, gently resuspended and flotated at 1000 *g* on Lymphoprep® Non-E-RFC were harvested at the interphase and washed twice SRBC in the pellet were lysed by autologous serum, and the lymphocytes (E-RFC enriched) were washed twice in BSS

Serological typing for HLA-DR determinants The technique was the standard complement dependent cytotoxicity (CDC) used for typing during the VIIth

HLA workshop 1977 (2) The T-cell suspension (E-RFC enriched) and the B-cell suspension (E-RFC depleted) were tested in parallel by a selected set of workshop VII antisera defining six DRw specificities Each specificity was defined by a minimum of two testsera; *DRw1:* sera Nos 09, 128, 152, *DRw2:* sera Nos. 14, 16, 17, 18, 21, 60, 81; *DRw3.* sera Nos 24, 25, 26, 27, 34, 35, 95, 133, 180, *DRw4* sera Nos. 37, 38, 104, 106, 129, *DRw7:* sera Nos 51, 52, 53, 54, 72; *DRw8*- sera Nos 58, 112 (2)

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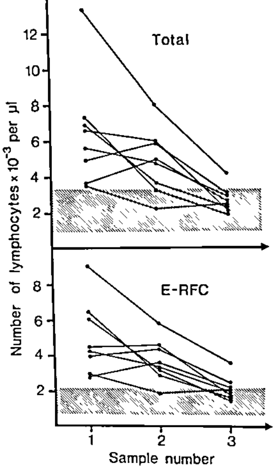


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2nd (1 week)	mean	7 5	4 8	78 6	15 4	4.4	9.3	53.9
	range	4 4–12 0	2 2–8 0	70–91	08–27	01–09	05–12	28–85
	SD	2 7	1 8	8 3	7 2	2 6	1 8	17.9
3rd (4–14 week)	mean	6 6	2 8	79 8	20 3	13 6	10.7	46.3
	range	5 5–8 1	1.9–4 2	69–87	13–24	08–25	06–23	24–63
	SD	1 0	0 7	7 5	3 7	5 1	6 7	15.9
Normal values (n = 35)	mean	6 65	2 1	71 0	22 1	11 5	17.3	52.2
	range	4.1–8 9	1 0–3 4	61–82	08–32	06–19	06–31	14–86
	SD	2 0	1.0	6.7	7.4	3.7	7.5	19.5

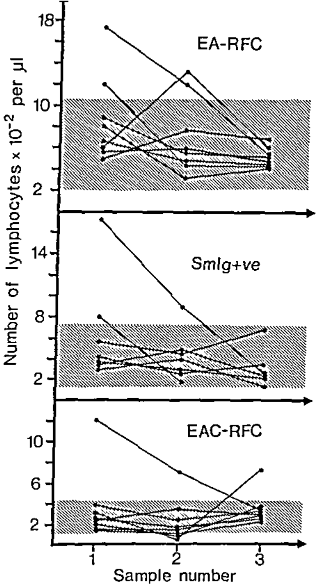


Fig. 2. Serial quantitations of EA-, EAC-RFC and SmIg positive lymphocytes in peripheral blood on patients with IM. The hatched area is normal range values from 35 normal donors.

were less than 2 per cent, as expected, due to the lymphocytosis which will reduce the relative number of monocytes. Normally the average contamination of monocytes in the isolated suspension of mononuclear cells is 2.9% (range 0–5%) due to use of defribrinated blood as blood source (9)

The relative yield of lymphocytes during the isolation from peripheral blood was calculated, and no changes were found for any sample compared to normal controls indicating that no selective loss of lymphocytes occurred (9).

Estimation of the absolute number of cells in the various lymphocyte subpopulations per µl peripheral blood gave results as shown in Fig. 1 and Fig. 2. In 7 of 8 patients tested the lymphocytosis was due only to E-RFC, i.e. T lymphocytes. The number of cells in the other subpopulations were within normal ranges. Only one patient showed an elevation of the total number of EA-, EAC-RFC and SmIg positive lymphocytes per µl blood in the acute phase, returning to normal values in the reconvalescence.

Identification of HLA-DR Antigens on the lymphocytes

HLA-DR typing during the acute phase and in the reconvalescense showed a pattern as depicted for two persons in Table 2. Not only B lymphocytes (non E-RFC) but also a substantial fraction of the T cells (E-RFC) were killed in the HLA-DR assay. This phenomenon was found in all IM patients tested during the acute phase of the disease. During reconvalescensce the patients were retested and showed the usual DR typing pattern, i.e. less than 10% killed lymphocytes in the T-cell suspension. Further, all testsera reacting positive with a given patient's B and T cells during IM reacted with the same donor's B cells after the disease. Sera identifying other HLA-DR specificities than those on lymphocytes of the actual patient consistently gave negative results (i.e. $<10\%$ killed T as well as B cells). These results indicate that HLA-DR determinants are expressed on a population of T cells in patients with IM.

Semiquantitation of DR + ve Lymphocytes in the T- and B-cell Fractions

The E-RFC enriched and depleted suspension used in the lymphocytotoxic assay were marked with surface membrane markers in six patients as shown in Table 3. In the T-cell suspension 78–95% E-RFC was found and less than 11% of the cells had B-cell membrane markers (complement receptors and SmIg). Only 0–15% of E-RFC were found in the B-cell suspension which contained nearly all SmIg + ve lymphocytes in a range of 58–75%.

Further the DR-typing resulted in 21–75% killed lymphocytes in the B-cell fraction and 31–75% cytolysis in the T-cell suspension Thus the number of lysed cells in the E-RFC enriched suspension exceeded the number of contaminating B cells. The semiquantitation (Table 3) is based on the average obtained with 3–12 positive testsera.

Absorption Experiments

Two of the patients were HLA-DRw7 + ve and serum No 054 (anti-DRw7) were titrated against their T and B cells and compared to the reactivity against two normal HLA-DRw7 + ve persons. Two experiments were performed each with one patient and one normal donor; both experiments gave identical results, and only results from one experi-

TABLE 2. *HLA-DR Typing of Two Patients in the Acute and the Reconvalescence Period. Subpopulations are Quantitated in the Suspensions Used for Typing*

		E-RFC %	EA-RFC %	EAC-RFC %	SmIg %	Anti-HLA-DRw1			Anti-HLA-DRw2					
						009[a]	128	152	014	016	017	021	060	081
Pt. No XII (HLA-DRw1/–)														
Acute phase	E-RFC depleted	0	55	27	60	•	••	•	–	–	–	–	–	–
	E-RFC enriched	89	06	01	<2	•	••	••	–	–	–	–	–	–
Reconv. period	E-RFC depleted	08	57	59	90	•	•••	•	–	–	–	–	–	–
	E-RFC enriched	94	07	01	<1	–	–	–	–	–	–	–	–	–
Pt. No XIII (HLA-DRw1/DRw2)														
Acute phase	E-RFC depleted	04	45	14	58	••	••	•	••	•••	••	•••	•••	••
	E-RFC enriched	95	04	<1	03	•••	••	••	••	•••	••	••	••	–
Reconv period	E-RFC depleted	06	61	45	93	•	••••	••••	••••	••••	••••	••••	••••	•••
	E-RFC enriched	78	04	<1	<2	–	–	–	–	–	–	–	–	–

– = <20% cytolyse
= 21–30% cytolyse
• = 31–50% cytolyse
•• = 51–75% cytolyse
••• = >76% cytolyse

[a] The VIIth HLA workshop serum number

TABLE 3 *Semiquantitation of DR + ve Cells in Suspensions Fractionated by E Rosetting. Based on the Average Reactivity of the Number of Positive Testsera Shown in Brackets*

Pt No			E-RFC %	EA-RFC %	EAC-RFC %	SmIg + ve %	DR2ve Semiquantitated
VIII	(HLA-DRw2/DRw7)	unseparated	70	36	02	18	
		E-RFC depleted	12	75	08	68	•••
		E-RFC enriched	90	22	00	11	••• (12)
X	(HLA-DRw4/–)	unseparated	81	10	08	07	
		E-RFC depleted	2	54	45	75	•••
		E-RFC enriched	82	3	<2	<1	•• (5)
XI	(HLA-DRw1/DRw7)	unseparated	73	27	07	08	
		E-RFC depleted	1	52	44	60	•••
		E-RFC enriched	78	8	<2	<1	••• (8)
XII	(HLA-DRw1/–)	unseparated	91	10	05	05	
		E-RFC depleted	0	55	27	60	•
		E-RFC enriched	89	6	<1	<2	•• (3)
XIII	(HLA-DRw1/DRw2)	unseparated	92	17	03	05	
		E-RFC depleted	4	45	14	58	••
		E-RFC enriched	95	4	<1	03	•• (10)
XVII	(HLA-DRw4/DRw7)	unseparated	83	25	04	11	
		E-RFC depleted	15	86	34	/	•••
		E-RFC enriched	90	12	<1	06	••• (8)

TABLE 4. *Reactivity of Serum No. 054 (anti HLA-DRw7) before and after Absorption with T Cells*

	Lymphocyte suspension	Anti HLA-DRw7 serum dilution 1	2	4	8	16	32	
Patient	E-RFC depleted	++++	+++	+++	++	+	–	Unabsorbed
	E-RFC enriched	+++	+++	+++	++	+	–	
Normal donor	E-RFC depleted	+++	+++	+++	++	+	–	
	E-RFC enriched	–	–	–	–	–	–	
Patient	E-RFC depleted	+++	+++	++	+	–	–	Absorbed with E-RFC enriched suspension from the normal person (30×10^6 cells/100 µl)
	E-RFC enriched	+++	++	++	+	–	–	
Normal donor	E-RFC depleted	+++	+++	++	+	–	–	
	E-RFC enriched	–	–	–	–	–	–	
Patient	E-RFC depleted	+	–	–	–	–	–	Absorbed with E-RFC enriched suspension from the patient (30×10^6 cells/100 µl)
	E-RFC enriched	–	–	–	–	–	–	
Normal donor	E-RFC depleted	–	–	–	–	–	–	
	E-RFC enriched	–	–	–	–	–	–	

Patient No. XI; HLA-DRw1/DRw7
Normal donor; HLA-DRw2/DRw7

ment is shown (Table 4). The titre against patient B and T cells was equal to the titre against the normal B cells. After absorption of the serum with normal T-cells, the strength of the serum was only reduced slightly. But an equal number of patients' T cells absorbed completely the activity against own T and B cells as well as the normal donor's B cells. Hence it is reasonable to conclude that the specificity of the »neoantigens« on the T cells during IM is identical to the specificity of the HLA-DR antigens on the B lymphocytes.

DISCUSSION

The present study demonstrates that the lymphocytosis in peripheral blood during the acute stage of IM is due to E-RFC, while the total numbers of EA-, EAC-RFC and SmIg positive lymphocytes are normal. Other studies have revealed an increase also in the B-cells, but this was only found for one patient in this study. An important technical factor may explain this discrepancy, i.e. »cold« autoantibodies against lymphocytes occur during IM (5, 16, 24, 27) resulting in an elevated percent of SmIg positive lymphocytes due to staining of cell-fixed autoantibodies (30). This factor should be eliminated by 37° C incubation of lymphocytes during the latex phagocytose test, resulting in elution of the cold reacting autoantibodies. On the other hand the EAC indicator cells identify the C3 receptors on the B cells, which is topographical near to the EBV receptor (13), and our estimates of EAC-RFC could be too small due to a steric hindrence from adherred EBV. However, this is unlikely as it has been impossible to identify EBV on the surface of peripheral lymphocytes of IM patients. Consequently we must conclude that the lymphocytosis seen in our patients is determined by T cells.

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The aim of this work was to investigate whether HLA-DR antigens could be identified on T lymphocytes during IM, and our results certainly support this hypothesis. The function of these cells, especially in relation to the possibility of suppressor T-cells as a limiting factor in IM is under investigation.

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	Lymphocyte suspension	Anti HLA-DRw7 serum dilution 1	2	4	8	16	32	
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	E-RFC enriched	***	***	***	**	*	–	
Normal donor	E-RFC depleted	***	***	***	**	*	–	
	E-RFC enriched	–	–	–	–	–	–	
Patient	E-RFC depleted	***	***	**	*	–	–	Absorbed with E-RFC enriched suspension from the normal person (30×10^6 cells/100 µl)
	E-RFC enriched	***	**	**	*	–	–	
Normal donor	E-RFC depleted	***	***	**	*	–	–	
	E-RFC enriched	–	–	–	–	–	–	
Patient	E-RFC depleted	*	–	–	–	–	–	Absorbed with E-RFC enriched suspension from the patient (30×10^6 cells/100 µl)
	E-RFC enriched	–	–	–	–	–	–	
Normal donor	E-RFC depleted	–	–	–	–	–	–	
	E-RFC enriched	–	–	–	–	–	–	

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CELL SURFACE MARKERS IN CORD BLOOD LEUCOCYTES AFTER STIMULATION WITH LIPOPOLYSACCHARIDE B

J SCHRÖDER, O TURUNEN, C LUNDQVIST and A DE LA CHAPELLE

Folkhälsan Institute of Genetics, Helsinki, and Department of Medical Genetics, University of Helsinki, Finland

Schroder, J, Turunen, O, Lundqvist, C & de la Chapelle, A Cell surface markers in cord blood leucocytes after stimulation with lipopolysaccharide B Acta path microbiol. scand Sect C, 86, 315–319, 1978

T- and B-lymphocyte markers were studied in cord blood cells cultured with lipopolysaccharide B (LPS) Cells cultured with leucoagglutinin (LA) and pokeweed mitogen (PWM) were used as controls LPS-induced lymphoblasts were negative for surface Ig, positive for intracellular Ig and did not form rosettes with sheep red blood cells (SRBC) LA-activated cells formed rosettes with SRBC, while PWM cultures showed a varying proportion of surface Ig-positive or SRBC rosetting cells, dependent on the time of culture About 50% of both LA- and LPS-activated lymphoblasts formed EA rosettes (specific for Fc receptors) and EAC rosettes (specific for complement receptors) The response of foetal cells to LPS was reduced when lymphocytes obtained from Isopaque-Ficoll gradients were passed through nylon wool columns, whereas this procedure led to an increased response to LA Thus LPS-activated foetal leucocytes are B lymphocytes expressing intracellular but not surface Ig

Key words Cord blood, lymphocyte culture, lipopolysaccharide B, surface immunoglobulin, intracellular immunoglobulin

J Schroder Folkhalsan Institute of Genetics, P O Box 819, SF-00101 Helsinki 10, Finland

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Lipopolysaccharide B (LPS) from *E coli* acts as a mitogen on B-lymphocytes of the mouse (1) Since the response generated is polyclonal, LPS has been termed a polyclonal B cell activator (PBA) (2) Lymphocytes from peripheral blood of human adults do not respond to LPS (8), except occasionally in cultures from patients with certain disorders (3) However, when stimulated with this mitogen, both spleen cells from human adults (5) and leucocytes from peripheral blood of human foetuses and neonates (umbilical cord) show a response measurable by thymidine uptake, blast transformation or mitotic division (8)

The aim of the present study was to ascertain whether the foetal cells activated by LPS are B lymphocytes or cells of some other category.

MATERIALS AND METHODS

Cells and culture Lymphocyte suspensions were obtained from cord blood in an Isopaque-Ficoll gradient by centrifugation Viable cells were counted in trypan blue and cultured on disposable polystyrene microplates (Cooke Microtitre® System Sterilin Ltd, Teddington, UK) Each well contained 1 to 5 × 10^5 cells in a total of 90% RPMI 1640 (Gibco Bio-Cult, Glasgow, Scotland) and 10% heat-inactivated pooled human AB serum L-glutamine (1 28 mM), penicillin (32IU/ml) and streptomycin (0 032 µg/ml) were added Cultures were incubated at 37°C in air containing 5% CO_2

Mitogens Crystallized leucoagglutinin (LA) from Pharmacia Fine Chemicals, Sweden, was used in a final concentration of 20 µg/ml of culture medium Lipopolysaccharide B (LPS) from *Escherichia coli* 055 B5 was purchased from Difco Laboratories, USA, and boiled in water bath for 2 h before use at a final concentration of

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J SCHRÖDER, O TURUNEN, C. LUNDQVIST and A DE LA CHAPELLE

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40 μg/ml. Pokeweed *mitogen (PWM)*, Grand Island Biological Company, USA, was used at a dilution of 1:100.

Cultures were harvested after 3, 5, 7 and 10 days, and cells were analysed for surface markers.

Surface markers. Surface Ig staining was carried out at 4°C as follows: 10^6 lymphocytes were incubated with a FITC-labelled polyvalent rabbit anti-human Ig (Behringwerke, Germany, diluted 1:16) for 15 min in the presence of 0.2% sodium azide. Cells were then washed once through a gradient of foetal calf serum (FCS) and slides were made with a cytocentrifuge (Cytospin Shandon Southern Instruments Ltd., England). Slides were fixed for 2 min in absolute ethanol and mounted in phosphate-buffered glycerol (nine parts glycerol, one part phosphate-buffered saline). For the detection of intracellular Ig in the lymphoblasts induced by LPS, cytocentrifuge slides were fixed in absolute acetone for 10 min at − 20°C, air dried, and subsequently stained with antihuman Ig (see surface Ig staining) diluted 1:20 for 15 min. Slides were rinsed in PBS and mounted in phosphate-buffered glucerol. The percentage of fluorescent cells (surface Ig, intracellular Ig) was determined, using a Lietz Ortholux II microscope equipped with epi-illumination.

T Lymphocytes were assayed by spontaneous rosette formation with sheep red blood cells (SRBC). 2×10^5 lymphocytes in 0.1 ml phosphate-buffered saline (PBS) + 20% FCS were incubated with 0.1 ml of a fresh 0.5% suspension of SRBC for 15 min at 37°C The cell suspension was then centrifuged at 200 *g* for 5 min at 5°C. After a 2-h incubation at 5°C the pellet was gently resuspended and diluted to 1 ml in PBS + 20% FCS

Cytocentrifuge slides were made and stained by the May-Grunwald-Giemsa technique The slides were used to identify the different categories of leucocytes and to evaluate the proportions of the different cell types forming rosettes A lymphocyte was scored as rosette-forming if it had three or more SRBC on its circumference

B lymphocytes carrying Fc receptors were identified by rosette formation with human 0 red blood cells (RBC) sensitized by rabbit anti-human 0 RBC serum (Ig G) (erythrocyte + antibody = EA) and B cells bearing complement receptors by rosette formation with human 0 RBC sensitized by rabbit anti-human 0 RBC serum (Ig M) and fresh human complement (erythrocyte + antibody + complement = EAC) 2×10^5 lymphocytes in 0.1 ml PBS + 1% bovine serum albumin (BSA) were incubated with 0.1 ml of a fresh 1% EA or EAC suspension at 18°C (EA) or 37°C (EAC) for 15 min. Cells were then centrifugated at 200 *g* for 5 min (at 18°C) and incubated for a further 15 minutes at 4°C (EA) or 18°C (EAC) Cytocentrifuge slides were made and stained with the May-Grunwald-Giemsa technique

Cells forming EA and EAC rosettes were determined in the same way as those forming SRBC rosettes.

Nylon wool columns. T cells were enriched in lymphocyte populations from umbilical cord blood by *filtration of cell* suspensions recovered from Isopaque-Ficoll gradients through nylon wool columns (6). The *depletion* of Ig positive cells was evaluated by staining suspensions before and after filtration with a FITC-labelled polyvalent rabbit anti-human Ig (see Methods surface Ig staining)

Lymphocyte populations from suspensions before and after filtration were then studied for responsiveness to the mitogens LPS and LA, the response being measured by the uptake of tritiated thymidine (see Methods: cells and culture).

RESULTS

Before culture with LPS, 23% of the cord blood lymphocytes were surface Ig-positive. This number decreased gradually until after 10 days no surface Ig-*positive cells could be detected* (Fig. 1). The numbers are mean values of five experiments. The variation of different experiments is *indicated* by vertical bars.

Since the response of cord blood cells to LPS occurs late, with a maximum at around 5–7 days of culture, there is a correlation between the increase in the number of LPS activated cells and the decrease of surface Ig-positive cells. Not a single surface Ig-positive lymphoblast was ever observed in these cultures, thus indicating that cells activated by LPS *did not form detectable amounts* of surface Ig.

In control cultures (without mitogen), the proportion of surface Ig-positive cells likewise dropped

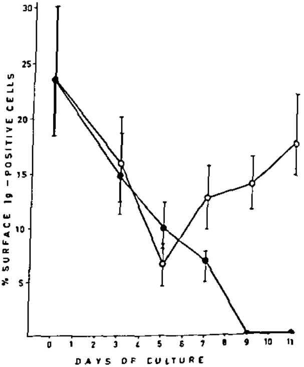


Fig 1 Surface Ig-positive cells in LPS cultures (●) and *controls (O)*

during the first five days, but then rose until on day 11 18% of the lymphocytes were surface Ig-positive (Fig. 1) (mean value of five experiments). This was not the result of proliferation of B lymphocytes in the control cultures, since no proliferating cells were observed, but was probably due to selective survival of subpopulations of B cells

After 11 days of culture, 0% of the lymphocytes in LPS cultures were surface Ig positive, while 18% of lymphocytes in control cultures had demonstrable amounts of surface Ig. This difference is highly significant.

After three days of culture with LPS, a few lymphoblasts positive for intracellular Ig could be detected, and with longer time of culture the number of lymphoblasts increased, as did those positive for intracellular Ig On day 7, when about 50% of the lymphocytes were transformed lymphoblasts, all showed detectable amounts of intracellular Ig, the fluorescence intensity ranging from weak to bright

Before culture, the proportion of lymphocytes forming rosettes with SRBC was about 60% (Table 1) After culture with LA for 3, 5 and 7 days, most cells were rosette-forming lymphoblasts (Table 1) After 10 days in LA culture, about 50% of the lymphocytes were rosette-forming lymphoblasts and more than 40% rosette-forming small lymphocytes In these cultures very few lymphocytes failed to form rosettes (Table 1).

Between cultures with and without LPS the difference in the frequency of lymphocytes forming rosettes with SRBC was small as compared with suspensions before culture (Table 1). However, both in LPS cultures and in controls without mitogen there was a clear increase in the number of macrophages, accompanied by an increase in the number of lymphoblasts.

After culture for seven days with LPS, 13% of all cells present (more than 50% of lymphocytes) were transformed. These LPS blasts were almost always non-rosetting, and the number of rosetting cells as a whole decreased with increasing culture time (Table 1). At the time of maximal response with LA, more than 80% of the lymphocytes were rosette-forming lymphoblasts, while at the time of maximal response to LPS, less than 5% of the lymphocytes were rosette-forming lymphoblasts. *This difference is highly significant. Identical* experiments carried out repeatedly have given *similar results, and the numbers in Table 1 are the* mean values of five experiments. Tables 2 and 3 (mean values of three experiments) show the frequency of cells from LA, LPS and control cultures forming EA and EAC rosettes. Roughly half the LA blasts and LPS blasts formed EA and EAC rosettes, the frequencies depending somewhat on the stage of culture, while the other half did not form rosettes.

The response to LPS decreased considerably when lymphocytes from an Isopaque-Ficoll gradient were depleted of B cells by passage through nylon wool columns (Fig. 2). By contrast, this procedure increased reactivity to LA (Fig 2) The counts are mean values of two experiments, and the variation

TABLE 1 *Percentage of Cells Forming Rosettes with SRBC in LA, LPS and Control Cultures*

	Day 0	Day 3			Day 5			Day 7			Day 10		
		control	LA	LPS	control	LA	LPS	control	LA	LPS	control	LA	LPS
Non-rosette-forming lymphoblasts	0	0	1	2	1	11	3	6	2	12	11	0	11
Rosette-forming lymphoblasts	0	1	80	1	0	78	0	0	91	1	0	47	1
Non-rosette-forming lymphocytes	35	32	7	12	31	3	9	20	6	8	33	6	35
Rosette-forming lymphocytes	50	39	7	32	36	3	9	19	0	2	0	42	0
Granulocytes	2	1	0	0	0	1	0	4	1	1	1	2	0
Monocytes or macrophages	13	27	5	53	32	4	79	51	0	76	55	3	53

40 µg/ml. Pokeweed mitogen (PWM), Grand Island Biological Company, USA, was used at a dilution of 1:100.

Cultures were harvested after 3, 5, 7 and 10 days, and cells were analysed for surface markers.

Surface markers. Surface Ig staining was carried out at 4°C as follows: 10^6 lymphocytes were incubated with a FITC-labelled polyvalent rabbit anti-human Ig (Behringwerke, Germany, diluted 1:16) for 15 min in the presence of 0.2% sodium azide. Cells were then washed once through a gradient of foetal calf serum (FCS) and slides were made with a cytocentrifuge (Cytospin Shandon Southern Instruments Ltd., England). Slides were fixed for 2 min in absolute ethanol and mounted in phosphate-buffered glycerol (nine parts glycerol, one part phosphate-buffered saline). For the detection of intracellular Ig in the lymphoblasts induced by LPS, cytocentrifuge slides were fixed in absolute acetone for 10 min at −20°C, air dried, and subsequently stained with antihuman Ig (see surface Ig staining) diluted 1:20 for 15 min. Slides were rinsed in PBS and mounted in phosphate-buffered glucerol. The percentage of fluorescent cells (surface Ig, intracellular Ig) was determined, using a Lietz Ortholux II microscope equipped with epi-illumination.

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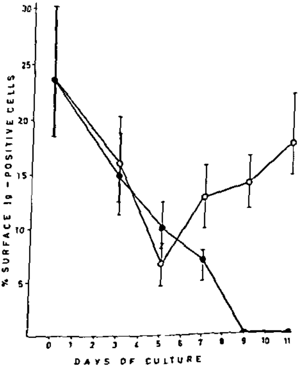


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Non-rosette-forming lymphocytes	35	32	7	12	31	3	9	20	6	8	33	6	35
Rosette-forming lymphocytes	50	39	7	32	36	3	9	19	0	2	0	42	0
Granulocytes	2	1	0	0	0	1	0	4	1	1	1	2	0
Monocytes or macrophages	13	27	5	53	32	4	79	51	0	76	55	3	53

TABLE 2. *Percentage of Cells Forming EAC Rosettes in LA, LPS and Control Cultures*

	Day 0	Day 3 control	Day 3 LA	Day 3 LPS	Day 5 control	Day 5 LA	Day 5 LPS	Day 7 control	Day 7 LA	Day 7 LPS	Day 10 control	Day 10 LA	Day 10 LPS
Non-rosette-forming lymphoblasts	0	0	31	1	2	67	12	0	48	17	6	23	12
Rosette-forming lymphoblasts	1	2	31	2	0	13	2	1	32	10	3	22	42
Non-rosette-forming lymphocytes	51	27	13	18	36	10	12	27	2	0	10	27	5
Rosette-forming lymphocytes	24	29	18	15	20	3	3	24	4	4	16	13	21
Granulocytes	0	0	1	0	0	0	0	0	0	0	0	0	0
Monocytes or macrophages	24	42	6	64	42	7	71	48	14	69	65	15	20

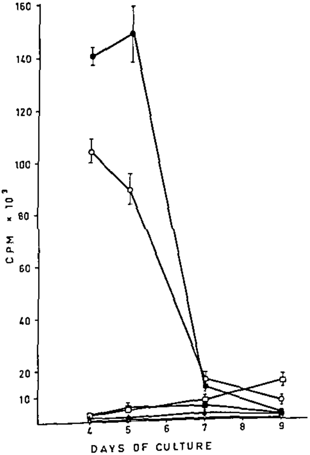


Fig. 2. Response to LA and LPS in lymphocytes obtained from Isopaque-Ficoll gradients and in cell populations enriched in T cells by passage through nylon wool and in controls. LA Isopaque-Ficoll, O; LA nylon wool, ●; LPS Isopaque-Ficoll, □; LPS nylon wool, ■; control Isopaque-Ficoll, ▲; control nylon wool, △.

between different experiments is indicated by vertical bars.

After 3, 5 and 7 days of culture with PWM, about 50% of all lymphocytes formed rosettes with SRBC. After 10 days of culture, most of the lymphocytes were non-rosetting. This probably reflected an increase in the number of B cells with longer culture time, since at the same time the frequency of surface Ig-positive cells had increased. In 5, 7 and 10-day PWM cultures, more than 50% of the lymphocytes were transformed lymphoblasts. Thus, the majority of cells scored as rosetting or surface Ig-positive were, in fact, blasts

DISCUSSION

Ringdén & Moller (5) have suggested that B lymphocytes from human spleen can be activated by LPS, a well-known mitogen of B cells in the mouse (1). We were unable to detect any surface Ig on human cord blood leucocytes activated by LPS, although these cells expressed intracellular Ig Since cord blood cells activated by LPS did not form rosettes with SRBC, it seems unlikely that these cells are T lymphocytes

This is also supported by the observation that populations depleted of B cells by passage through nylon wool showed a considerably weaker response to LPS but a stronger response to LA.

TABLE 3. *Percentage of Cells Forming EA Rosettes in LA, LPS and Control Cultures*

	Day 0	Day 3 control	Day 3 LA	Day 3 LPS	Day 5 control	Day 5 LA	Day 5 LPS	Day 7 control	Day 7 LA	Day 7 LPS	Day 10 control	Day 10 LA	Day 10 LPS
Non-rosette-forming lymphoblasts	0	0	32	1	0	60	12	1	34	10	2	17	4
Rosette-forming lymphoblasts	0	0	25	1	0	23	3	2	25	11	4	11	18
Non-rosette-forming lymphocytes	47	27	10	16	31	4	14	14	6	3	5	26	3
Rosette-forming lymphocytes	41	30	25	15	25	8	7	12	8	1	11	9	18
Granulocytes	0	0	0	1	0	0	0	0	0	0	0	0	0
Monocytes or macrophages	12	43	8	66	44	5	64	71	27	75	78	37	57

Since both LPS- and LA-activated cells formed EA and EAC rosettes, these assays are evidently not specific for B lymphocytes. This is supported by a recent report indicating that Fc and complement receptors are expressed on PHA activated T lymphocytes (7).

Thus there seem to be only three valid criteria for distinguishing between mitogen-activated T and B cells: rosetting with SRBC, and the presence of surface or intracellular Ig. Even so, however, our results are inconsistent, since in our hands LPS activated cord blood cells are both SRBC and surface Ig negative. The fact that surface Ig positive lymphoblasts could be found in PWM cultures from cord blood rules out the possibility that activated B lymphocytes from cord blood are always surface Ig-negative. However, the fact that LPS-activated cord blood cells express intracellular Ig shows clearly that they are B lymphocytes. Since they do not express surface Ig as PWM-activated B cells, it seems reasonable to assume that the surface Ig has such a fast turn-over rate in these cells that they appear to be surface Ig negative even in the presence of azid (4).

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